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(57) Abstract

The invention provides a means of modulating cell motility and/or invasiveness by regulating interactions between $\alpha 6\beta 4$ integrin and actin, and by regulating the biological activity of signaling molecules comprising a phosphoinositide 3–hydroxyl kinase (PI 3–K) signaling pathway, such as PI 3–K, and by regulating the biological activity of protein kinase C (PKC), and cAMP phosphodiesterase. Methods for the isolation of compounds that modulate cell invasiveness by regulating the biological activity of $\alpha 6\beta 4$ integrin/actin interactions and PI 3–K, PKC, and cAMP phosphodiesterase signaling pathways also are provided.

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METHODS AND REAGENTS FOR MODULATING CELL MOTILITY

Statement as to Federally Sponsored Research

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Field of the Invention

The field of the invention is regulation of cell motility and invasion in wound healing and cancer.

Background of the Invention

α6β4 integrin is essential for the organization and maintenance of epithelial sheet architecture. In many epithelia, α6β4 integrin mediates the formation of stable adhesive structures, termed hemidesmosomes, which link the intermediate filament cytoskeleton with the extracellular matrix. The ability of α6β4 integrin to associate with intermediate filaments distinguishes it from other integrins that interact primarily with the actin cytoskeleton.

Although epithelial cells are typically anchored in place via their cell-cell adhesion receptors and α6β4 integrin-containing hemidesmosomes, epithelial cells also can lose their anchoring contacts and acquire a motile, mesenchymal phenotype. The acquisition of motility is involved in both physiologically beneficial and pathological processes: for example, epithelial cell migration is an integral component of both wound healing and invasive carcinoma.

Acquisition of a motile phenotype involves alterations in the expression and function of surface receptors that maintain the normal stationary epithelial phenotype. For example, invasive carcinoma is characterized by the loss of functional cell-cell adhesion receptors, such as cadherins. In contrast, α6β4 integrin persists in many tumor cell types that exhibit a motile phenotype. This observation is counterintuitive, given that α6β4 integrin is found in hemidesmosomes, which are not commonly observed in motile cells such as invasive carcinoma cells.

Relatively little is known about integrin specificity and integrin-mediated signaling events in motile cells. Downstream signaling pathways that are activated by α6β4 integrin and contribute to motility may differ from signaling pathways regulated by other integrins, especially given the size and structural diversity of the cytoplasmic domains among the various β4 isoforms. Hence, it would be desirable to identify molecules that comprise such a signaling pathway. Such molecules may provide important biological targets for inhibiting tumor invasiveness, and for stimulating wound healing.

Summary of the Invention

The α6β4 integrin has been implicated in the promotion of invasive carcinoma. We have discovered that the downstream signaling pathway through which α6β4 integrin promotes cell motility and invasiveness requires activation of phosphoinositide 3-hydroxyl kinase (PI 3-K) and its downstream effector, Rac.

Furthermore, members of the protein kinase C (PKC) family, some of which are downstream effectors of PI 3-K, are involved in this signaling process, as is cAMP phosphodiesterase. In contrast, activation of other downstream effectors

of PI 3-K, such as Akt kinase, inhibits cell invasiveness, perhaps by competing for substrates of PI 3-K.

We have also observed a novel interaction between α6β1 integrin and actin in several lines of invasive carcinoma cells, and in cells that become motile in an *in vitro* wound model.

The invention features methods for modulating the motility or invasiveness of a cell, and methods for identifying compounds that modulate the motility or invasiveness of a cell, by regulating the biological activity of the molecules comprising a PI 3-K-dependent signaling pathway that regulates cell motility and/or invasiveness, and by regulating PKC, cAMP phosphodiesterase, and/or α6β1 integrin/actin interactions.

In a first aspect, the invention features a method of identifying a compound that modulates the motility or invasiveness of a cell, comprising the steps of: (a) exposing a sample to a test compound, wherein the sample includes PI 3-K, PKC, cAMP-PDE, or α6β4 integrin, and (b) assaying for altered biological activity of PI 3-K, PKC, cAMP-PDE, or α6β4 integrin. A decrease in the biological activity, relative to the biological activity of a sample not exposed to the compound, indicates a compound that decreases cell motility or cell invasiveness, and an increase in the biological activity, relative to the biological activity of a sample not exposed to the compound, indicates a compound that increases cell motility or cell invasiveness.

In various embodiments of the first aspect of the invention, the PI 3-K, PKC, or cAMP-PDE may be constitutively activated, the PI 3-K, PKC, cAMP-PDE, or α6β4 integrin may be within a cell, the sample may comprise cell lysate or cell extract, the PKC may be an atypical PKC, or the method may further comprise a step in which the level of α6β4 integrin or the biological

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activity of PI 3-K, PKC, cAMP-PDE, or α6β4 integrin is increased or decreased prior to exposing the sample to the test compound.

In a second aspect, the invention features a method of identifying a compound that modulates the motility or invasiveness of a cell, comprising the steps of: (a) modulating the biological activity of PI 3-K, PKC, cAMP-PDE, Akt, or α6β4 integrin in a cell, (b) exposing the cell to a test compound, and (c) assaying for altered motility or invasiveness of the cell exposed to said compound. A decrease in the motility or the invasiveness, relative to the motility or invasiveness of a cell not exposed to the compound, indicates a compound that decreases cell motility or cell invasiveness, and an increase in the motility or the invasiveness, relative to a cell not exposed to said compound, indicates a compound that increases cell motility or cell invasiveness.

In various embodiments of the second aspect of the invention, the biological activity may be increased by introducing constitutively active PI 3-K, PKC, or cAMP-PDE into the cell, the biological activity may be decreased by introducing dominant-negative PI 3-K, PKC, or cAMP-PDE into the cell, or the PKC may be an atypical PKC.

In a third aspect, the invention features a method of decreasing the invasiveness of a cell or decreasing the predisposition to developing an invasive cell, comprising identifying the presence of at least one invasive cell or at least one cell with a predisposition to developing invasiveness, and exposing at least one invasive cell or at least one cell with a predisposition to developing invasiveness to a compound that decreases the biological activity of PI 3-K, PKC, cAMP-PDE, or α6β4 integrin, or increases the biological activity of Akt.

In various embodiments of the third aspect of the invention, the PKC may be an atypical PKC. The compound may be: an antibody that specifically binds PI 3-K, PKC, cAMP-PDE, or α6β4 integrin; antisense nucleic acid that specifically hybridizes with nucleic acid encoding PI 3-K, PKC, or cAMP-PDE; dominant-negative PI3-K, dominant-negative PKC, dominant-negative cAMP-PDE, or constitutively-activated Akt; wortmannin; or a substrate for PI 3-K, PKC, or cAMP-PDE.

In other embodiments of the third aspect of the invention, the cell may be a neoplastic cell, such a colon carcinoma cell, a breast carcinoma cell, a prostate carcinoma cell, a cervical carcinoma cell, a uterine carcinoma cell, a testicular carcinoma cell, a liver carcinoma cell, an ovarian carcinoma cell, a renal carcinoma cell, a bladder carcinoma cell, a lung carcinoma cell, a laryngeal carcinoma cell, a squamous carcinoma cell, or a salivary gland carcinoma cell.

In a fourth aspect, the invention features a method of increasing the motility of a cell, comprising exposing the cell to a compound that increases the biological activity of PI 3-K, PKC, cAMP-PDE, or α6β4 integrin.

In various embodiments of the fourth aspect of the invention, increasing the motility of the cell may result in increased wound healing; the PKC may be an atypical PKC; the cell may be an epithelial cell, such as an epidermal epithelial cell, an oral epithelial cell, a nasal epithelial cell, a gastrointestinal epithelial cell, a rectal epithelial cell, or an anal epithelial cell; and the wound may result from a gastric ulcer, a duodenal ulcer, inflammatory bowel disease, ulcerative colitis, Crohn's disease, hemorrhoids, surgery, cancer, irradiation, exposure to toxic compounds, or physical trauma.

By "invasiveness" and/or "motility" is meant the relative ability of a cell to migrate through a substratum. The substratum may be artificial (such

as Matrigel) or natural (such as an extracellular matrix laid down by cells). Invasiveness and/or motility are measured by motility assays known to those skilled in the art, such as the Matrigel invasion assay described herein. The relative motility and/or invasiveness of a cell is expressed in comparison to a reference cell. For example, as shown in Fig. 2A, MDA-MB-435 cells expressing β4 integrin are 3-4-fold more invasive than parental cells not expressing β4 integrin.

By "neoplastic" is meant a cell or tissue multiplying or growing in an abnormal manner. Neoplastic growth is uncontrolled and progressive, and occurs under conditions that would not elicit, or would cause cessation of, multiplication of normal cells.

By "wound" is meant an injury to the body (as from surgery, physical trauma, injury, illness, disease, or exposure to excessive radiation or toxic compounds) that involves an interruption or laceration of an epithelium (e.g., epidermal epithelium or the epithelial lining of the oral, nasal, or anal/rectal mucosa or gastrointestinal tract).

By "wound healing" is meant the biological processes, including epithelial cell migration, that occur during repair of an injury to the body.

By "phosphoinositide 3-hydroxyl kinase" or "PI 3-K" is meant a lipid kinase, preferably derived from an animal, most preferably a mammal (such as a human or rodent), that has the ability to enzymatically phosphorylate phosphoinositide lipids at the D3 position, and whose activity stimulates cell motility and/or invasiveness, as described herein.

By "protein kinase C" or "PKC" is meant a member of a family of serine/threonine protein kinases, preferably derived from an animal, most preferably a mammal (such as a human or rodent), that are involved in signal transduction pathways that regulate many biological processes, including, but

not limited to, growth and differentiation. The biological activity (e.g., kinase activity) of "conventional" PKCs is known in the art to be activated by Ca^{+2}, phospholipid, diacylgycerol, and phorbol ester. In contrast, it is known that the "novel" PKC subfamily members (δ, ϵ, η, and θ isoforms) and the "atypical" subfamily members (ζ, ι and λ isoforms) are not activated by Ca^{-2}. Furthermore, biological activity of the atypical PKCs is independent of diacylglycerols and phorbol esters (see the Transduction Laboratories 1997 Antibody Catalog, p. 136-141). Certain members of the PKC family have the ability to act as downstream effectors in PI 3-K-mediated signaling pathways. These include, and are not limited to, the ϵ, ζ, ι and λ isoforms of PKC.

By "cAMP phosphodiesterase" or "cAMP-PDE" is meant an enzyme, preferably derived from an animal, most preferably a mammal (such as a human or rodent), that hydrolyzes cyclic AMP, yielding AMP plus a proton.

By "biological activity of PI 3-K," "biological activity of PKC," or "biological activity of cAMP-PDE," is meant, respectively, the enzymatic action of PI 3-K, PKC, or cAMP-PDE. Alterations in biological activity of PI 3-K, PKC, or cAMP-PDE may be determined by detecting or measuring enzymatic activity of PI 3-K, PKC, or cAMP-PDE in a test sample and comparing it to the analogous enzymatic activity in a reference sample, using assays that are known in the art or disclosed herein.

Alterations in biological activity of PI 3-K, PKC, or cAMP-PDE may also be determined by comparing PI 3-K, PKC, or cAMP-PDE polypeptide levels, mRNA levels, or reporter gene (under the regulation of a PI 3-K, PKC, or cAMP-PDE transcriptional control element) activity levels in a test sample to polypeptide levels, mRNA levels, or reporter gene levels in an appropriate reference sample.

By "biological activity of α6β4 integrin" is meant the interaction of α6β4 integrin with actin. Alterations in biological activity of α6β4 integrin may be determined by detecting or measuring the amount of interaction between α6β4 integrin and actin in a test sample and comparing it to the amount of α6β4 integrin/actin interaction in an appropriate reference sample, using assays that are known in the art or disclosed herein.

The amount of PI 3-K, PKC, cAMP-PDE, or α6β4 integrin biological activity may be modulated by increasing or decreasing the number of PI 3-K, PKC, cAMP-PDE, or α6β4 polypeptide molecules present intracellularly, by stimulating or inhibiting upstream modulators or downstream effectors of PI 3-K, PKC, cAMP-PDE, or α6β4 within the PI 3-K signal transduction pathway(s) that regulate(s) cell motility and/or invasiveness, or by post-translationally modifying PI 3-K, PKC, cAMP-PDE, or α6β4. For example, phosphorylation of PI 3-K stimulates its biological activity.

Biological activity may be less than or greater than the activity of wild-type PI 3-K, PKC, cAMP-PDE, or α6β4. For example, as shown in the Examples below, constitutively active PI 3-K has more than 100% of wild-type PI 3-K activity, and dominant-negative PI 3-K has less than 100% of wild-type activity. The absence of biological activity is defined by the presence of less than 10% of the biological activity that is found when assaying the wild-type protein in the same relevant assay.

By "high stringency conditions" is meant conditions that allow hybridization comparable with the hybridization that occurs during an overnight incubation using a DNA probe of at least 500 nucleotides in length, in a solution containing 0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA (fraction V), and 100 μg/ml denatured, sheared salmon sperm DNA, at a temperature of 65° C, or a solution containing 48% formamide, 4.8X SSC (150 mM NaCl, 15 mM trisodium citrate), 0.2 M Tris-Cl, pH 7.6, 1X Denhardt's

solution, 10% dextran sulfate, 0.1% SDS, and 100 µg/ml denatured, sheared salmon sperm DNA, at a temperature of 42° C (these are typical conditions for high stringency Northern or Southern, or colony hybridizations). High stringency hybridization may be used for techniques such as high stringency PCR, DNA sequencing, single strand conformational polymorphism analysis, and *in situ* hybridization. The immediately aforementioned techniques are usually performed with relatively short probes (e.g., usually 16 nucleotides or longer for PCR or sequencing, and 40 nucleotides or longer for *in situ* hybridization). The high stringency conditions used in these techniques are well known to those skilled in the art of molecular biology, and may be found, for example, in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997, hereby incorporated by reference.

By "probe" or "primer" is meant a single-stranded DNA or RNA molecule of defined sequence that can base pair to a second DNA or RNA molecule that contains a complementary sequence (the "target"). The stability of the resulting hybrid depends upon the extent of the base pairing that occurs. The extent of base-pairing is affected by parameters such as the degree of complementarity between the probe and target molecules, and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as temperature, salt concentration, and the concentration of organic molecules such as formamide, and is determined by methods known to one skilled in the art. Probes or primers specific for nucleic acids (e.g. PI 3-K nucleic acid) preferably will have at least 35% sequence identity, more preferably at least 45-55% sequence identity, still more preferably at least 60-75% sequence identity, still more preferably at least 80-90% sequence identity, and most preferably 100% sequence identity. Probes may be detectably-labelled, either radioactively, or non-radioactively, by methods well-known to those skilled in the art. Probes are used for methods

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involving nucleic acid hybridization, such as: nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, Northern hybridization, in situ hybridization, electrophoretic mobility shift assay (EMSA).

By "pharmaceutically acceptable carrier" means a carrier which is physiologically acceptable to the treated mammal while retaining the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington's Pharmaceutical Sciences, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA.

By "identity" is meant that a polypeptide or nucleic acid sequence possesses the same amino acid or nucleotide residue at a given position, compared to a reference polypeptide or nucleic acid sequence to which the first sequence is aligned. Sequence identity is typically measured using sequence analysis software with the default parameters specified therein, such as the introduction of gaps to achieve an optimal alignment (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably 85%, more preferably 90%, and most preferably 95% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the

length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

By "substantially pure polypeptide" is meant a polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure polypeptide may be obtained, for example, by extraction from a natural source (e.g., cultured cells) by expression of a recombinant nucleic acid encoding the polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides not only includes those derived from eukaryotic organisms but also those synthesized in *E. coli* or other prokaryotes.

By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA

or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transformation" is meant any method for introducing foreign molecules into a cell (e.g., a bacterial, yeast, fungal, algal, plant, insect, or animal cell). Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion, calcium phosphate precipitation, transduction (e.g., bacteriophage, adenoviral, or retroviral delivery), electroporation, and biolistic transformation are just a few of the methods known to those skilled in the art which may be used.

By "transformed cell," "transfected cell," or "transduced cells" is meant a cell (or a descendant of a cell) into which a DNA molecule encoding a polypeptide, for example, a dominant-negative PI 3-K, has been introduced, by means of recombinant DNA techniques.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., a polypeptide, a recombinant protein or a RNA molecule).

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific, temporal-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' or intron sequence regions of the native gene.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

By "detectably-labelled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule. Methods for detectably-labelling a molecule are well known in the art and include, without limitation, radioactive labelling (e.g., with an isotope such as ^{32}P or ^{35}S) and nonradioactive labelling (e.g., chemiluminescent labelling, e.g., fluorescein labelling).

By "antisense" as used herein in reference to nucleic acids, is meant a nucleic acid sequence that is complementary to the coding strand of a gene, for example, a PI 3-K, PKC, or cAMP-PDE gene. An antisense nucleic acid is capable of preferentially lowering the activity of a polypeptide encoded by a gene for which the antisense nucleic acid contains a complementary nucleic acid sequence.

By "specifically binds" is meant an antibody that recognizes and binds a human polypeptide (e.g., PI 3-K, PKC, cAMP-PDE, α6β4 integrin) but that does not substantially recognize and bind other (non-PI 3-K) molecules in a sample, e.g., a biological sample, that naturally includes protein.

By "neutralizing antibodies" is meant antibodies that interfere with any of the biological activities of a polypeptide (such as PI 3-K, PKC, cAMP-PDE, or α6β4 integrin); for example, the ability of PI 3-K to catalyze the phosphorylation of phosphoinositide lipids. The neutralizing antibody reduces the biological activity of PI 3-K, PKC, cAMP-PDE, or α6β4 integrin preferably by at least 25%, more preferably by at least 50%, yet more preferably by at least 70%, and most preferably by at least 90%. Any standard assay for the biological activity of PI 3-K, PKC, cAMP-PDE, or α6β4 integrin may be used to assess potentially neutralizing antibodies that are specific for these polypeptides.

By "expose" is meant to allow contact between an animal, cell, lysate or extract derived from a cell, or molecule derived from a cell, and a test compound.

By "treat" is meant to submit or subject an animal (e.g. a human), cell, lysate or extract derived from a cell, or molecule derived from a cell to a test compound.

By "test compound" is meant a chemical, be it naturally-occurring or artificially-derived, that is surveyed for its ability to modulate an alteration in reporter gene activity or protein levels, by employing one of the assay methods described herein. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

By "assaying" is meant analyzing the effect of a treatment, be it chemical or physical, administered to whole animals, cells derived therefrom, cell lysates or extracts, or partially- or fully-purified molecules from cell lysates or extracts. The material being analyzed may be an animal, a cell, a lysate or extract derived from a cell, or a molecule derived from a cell. The analysis may be for the purpose of detecting altered cell motility and/or invasiveness, altered protein biological activity (e.g., altered PI 3-K, PKC, or cAMP PDE enzymatic activity, or altered interactions of $\alpha 6\beta 4$ integrin with actin), altered protein stability, altered protein levels, altered mRNA levels, altered gene expression, or altered mRNA stability. The means for analyzing may include, for example, cell invasion assays, detection or quantification of the product of an enzymatic reaction (e.g., the formation of phosphorylated lipids as a result of PI 3-K activity), antibody-mediated detection or quantification of protein, immunoprecipitation, detection or quantification of mRNA by methods such as reverse transcription-polymerase chain reaction (RT-PCR) or filter hybridization (e.g., Northern or dot-blotting).

By "modulating" is meant changing, either by decrease or increase, the relative invasiveness of a cell, or the biological activity of PI 3-K, PKC, cAMP PDE, or a polypeptide in the PI 3-K-dependent pathway that mediates α6β4 integrin-stimulated cell invasiveness.

By "a decrease" is meant an inhibition of the motility and/or invasiveness of a cell, or an inhibition of the level of biological activity of PI 3-K, PKC, cAMP PDE, α6β4 integrin or any other polypeptide in the PI 3-K-dependent pathway that mediates α6β4 integrin-stimulated cell, as measured by a lowering in: a) the motility or invasiveness of a cell in an assay known to those skilled in the art, e.g., a Matrigel invasion assay; b) biological activity of a polypeptide in the PI 3-K-dependent pathway (e.g., the phosphorylation of inositide lipids by PI 3-K); c) relative protein (or phosphorylated protein) levels, as measured by ELISA; d) reporter gene activity, as measured by reporter gene assay, for example, lacZ/β-galactosidase, green fluorescent protein, luciferase, etc., under the transcriptional regulation of a PI 3-K, PKC, or cAMP-PDE transcriptional control element; or e) mRNA levels, as measured by PCR relative to an internal control, for example, a "housekeeping" gene product such as β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In all cases, the lowering is preferably by at least 25% more preferably by at least 40%, still more preferably by at least 50%, and even more preferably by at least 70%. Most preferably, the lowering is by at least 80%.

By "an increase" is meant a stimulation of the motility and/or invasiveness of a cell, or in the level of biological activity of PI 3-K, PKC, cAMP PDE, or a polypeptide in the PI 3-K-dependent pathway that mediates α6β4 integrin-stimulated cell invasiveness, as measured by a rise in: a) the motility or invasiveness of a cell in an assay known to those skilled in the art, e.g., a Matrigel invasion assay; b) biological activity of a polypeptide in the PI 3-K-dependent pathway (e.g., the phosphorylation of inositide lipids by PI 3-

K); c) relative protein (or phosphorylated protein) levels, as measured by ELISA; d) reporter gene activity, as measured by reporter gene assay, for example, lacZ/β-galactosidase, green fluorescent protein, luciferase, etc., under the transcriptional regulation of a PI 3-K, PKC, or cAMP PDE transcriptional control element; or e) mRNA levels, as measured by PCR relative to an internal control, for example, a "housekeeping" gene product such as β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In all cases, the rise is preferably by at least 25% more preferably by at least 40%, still more preferably by at least 50%, and even more preferably by at least 70%. Most preferably, the rise is by at least 80%.

By "alteration in the level of gene expression" is meant a change in gene activity such that the amount of a product of the gene, i.e., mRNA or polypeptide, is increased or decreased, or that the stability of the mRNA or the polypeptide is increased or decreased. Alterations in the level of gene expression may be detected or measured by comparing polypeptide levels, nucleic acid levels, or reporter gene (under the transcriptional regulation of transcriptional control elements from the gene of interest) activity in a test sample to that of a reference sample.

By "reporter gene" is meant any gene that encodes a product whose expression is detectable and/or quantitatable by immunological, chemical, biochemical or biological assays. A reporter gene product may, for example, have one of the following attributes, without restriction: fluorescence (e.g., green fluorescent protein), enzymatic activity (e.g., lacZ/β-galactosidase, luciferase, chloramphenicol acetyltransferase), toxicity (e.g., ricin A), or an ability to be specifically bound by a second molecule (e.g., biotin or a detectably-labelled antibody). It is understood that any engineered variants of reporter genes, which are readily available to one skilled in the art, are also included, without restriction, in the forgoing definition.

By "protein" or "polypeptide" or "polypeptide fragment" is meant any chain of more than two amino acids, regardless of post-translational modification (e.g., glycosylation or phosphorylation), constituting all or part of a naturally-occurring polypeptide or peptide, or constituting a non-naturally occurring polypeptide or peptide.

Brief Description of the Drawings

Fig. 1 is a diagram showing a FACS analysis of surface expression of integrin subunits in MDA-MB-435 transfectants.

Fig. 2A is a graph showing the relative invasiveness of MDA-MB-435 β4 and β4-ΔCYT transfectants.

Fig. 2B is a graph showing the relative invasiveness of a MDA-MB-435 β4 transfectant.

Fig. 3A is a graph showing an analysis of MAPK, PI 3-K, and p70 S6K involvement in MDA-MB-435 invasion. Panel A shows a Matrigel invasion assay, and Panel B shows a Western blot.

Fig. 3B is an autoradiogram showing MAPK activity of MDA-MB-435 β4 transfectants under various conditions.

Fig. 4A is an autoradiogram of a kinase assay showing PI 3-K activity in MDA-MB-435 β4 transfectants.

Fig. 4B is a diagram showing PI 3-K activity in MDA-MB-435 β4 and β4-ΔCYT transfectants.

Figs. 5A and 5B are graphs showing analyses of PI 3-K involvement in invasion of MDA-MB-435 cells by transient transfections.

Figs. 6A-6D are graphs showing analyses of downstream effectors in PI 3-K-dependent invasion of MDA-MB-435 cells.

Fig. 7A is a graph showing that α6β4 integrin mediates invasiveness of an invasive carcinoma cell line.

Fig. 7B is a graph showing that PI 3-K mediates lamellar formation in an invasive colon carcinoma cell line.

Fig. 7C is a series of photomicrographs showing that lamellar formation of an invasive colon carcinoma cell line is dependent upon α6β4 integrin and PI 3-K.

Figs. 8A and 8B are, respectively, an autoradiogram of a kinase assay and a graph depicting analyses of PI 3-K activity in Clone A cells.

Figs. 9A-9C are graphs showing that the PKC inhibitor Calphostin C inhibits the invasiveness of β4 integrin-transfected MDA-MB-435 cells, whereas the tumor promoter PMA does not.

Fig. 10 is a graph showing that β4 integrin-transfected MDA-MB-435 clones (3A7, 5B3) have higher phosphodiesterase activity that mock-transfected cells (6D7).

Fig. 11 is a graph showing that the phosphodiesterase inhibitor IBMX blocks the LPA-stimulated chemotaxis of MDA-MB-435 cells.

Figs.12A-12F are photomicrographs showing that α6β4 integrin co-localized with F-actin in filapodia of clone A cells on laminin-1.

Figs. 13A-13D are photomicrographs of *in situ*-detergent-extracted cells, showing that α6β1 integrin remains colocalized with F-actin.

Fig. 14 is an immunoprecipitation-western blot showing that α6β1 integrin is released from permeabilized clone A cells by the actin-severing protein gelsolin.

Figs. 15A-15E are photomicrographs of immunostained cells showing that α6β4 integrin is localized in actin-containing motility structures of various carcinoma cell lines.

Fig. 15F is a graph showing that an α6 integrin-specific antibody inhibits formation of lamallae in carcinoma cells.

Fig. 16A-16F are photomicrographs showing that lamallar extension during T84 cell wound healing is wortmannin-sensitive.

Detailed Description of the Invention

We have established that the coupling of a specific integrin, α6β4 integrin, to the phosphoinositide 3-hydroxylkinase (PI 3-K) signaling pathway promotes the invasion of carcinoma cells. Furthermore, we have observed that PI 3-K plays a role in wound-induced cell motility, suggesting that activation of PI 3-K dependent signaling is necessary for cell migration. Our experiments show that α6β4 activates PI 3-K more effectively than do α6β1 and other β1 integrins, thereby demonstrating specificity in integrin-mediated activation of the PI 3-K pathway. An essential role for PI 3-K in cell migration constitutes a novel function for this kinase and implies that downstream effectors of PI 3-K are critical for cell motility and invasion. In support of this statement, we provide evidence that the small GTP-binding protein Rac is downstream of PI 3-K in the cells examined and that Rac is involved in invasion.

Unlike Rac, the Akt and S6K serine/threonine kinases do not contribute to the invasive process. This is surprising because both Akt and S6K are regulated by PI 3-K and are activated by the α6β4 integrin. However, we found that activation of members of the protein kinase C serine/threonine kinase family, particularly the atypical isoforms, also are necessary for cell motility. We further noted increased cAMP phosphodiesterase activity in β4 integrin-activated cell motility. Finally, we observed a novel interaction between α6β4 integrin and F-actin in motile cells. This finding was surprising, as α6β4 integrin is not thought to interact with the actin cytoskeleton, but instead, with intermediate filaments such as cytokeratins. Collectively, our findings provide a mechanism for the involvement of α6β4 in promoting cell motility and invasiveness and reveal that essential elements of this process

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include a PI 3-K signaling pathway, as well as other signaling molecules, and a novel α6β4-integrin/actin interaction.

Invasion is a defining event in the progression of carcinoma. In general, the invasion process represents the ability of epithelial cells to acquire a mesenchymal phenotype characteristic of the breast and colon carcinoma cells used in our experiments.

To arrive at our current discoveries, we initially showed that expression of α6β4 integrin increased the invasion of both colon and breast carcinoma cells. Next, we established a mechanism for this increase by demonstrating that the α6β4 integrin activates a PI 3-K signaling pathway in carcinoma cells that is necessary for the invasive ability of these cells and that this α6β4-mediated pathway is linked to cell motility. Although protease activity is essential for invasion, no differences in protease expression or localization were observed between mock- and β4-transfectants of MDA-MB-435 breast carcinoma cells. Based on these findings, we conclude that the α6β4 integrin is critical for invasion because it promotes carcinoma motility through a PI 3-K-dependent pathway.

A central role for PI 3-K and its lipid products in carcinoma progression is indicated by our findings that wortmannin and dominant-interfering p85 subunits of PI 3-K inhibited invasion of MDA-MB-435 cells and that a constitutively active p110 subunit of PI 3-K increased their invasion. We provide evidence that integrins differ in their ability to activate PI 3-K, based on the preferential activation of PI 3-K by α6β4, compared to β1 integrins. This difference, observed in both MDA-MB-435 breast carcinoma cells and clone A colon carcinoma cells, is linked to a specific cellular response.

Our data regarding the PI 3-K-dependent formation of lamellae in clone A colon carcinoma cells indicate that the localized regulation of PI 3-K

activity by α6β4 integrin provides an efficient mechanism for targeting downstream functional effects of this kinase. The migration of invasive carcinoma cells, and of motile colon epithelial cells in an *in vitro* wound healing model, involves the dynamic formation of actin-containing motility structures such as lamellae and filopodia. An important finding is that the formation of these lamellae is dependent on PI 3-K. Quite surprisingly, wortmannin inhibited the formation of lamellae but had little effect on the adhesion of these cells. These observations implicate a specific role for α6β4-regulated PI 3-K activity in inducing the formation of actin-containing motility structures in carcinoma cells, and in wound-closing by epithelial cells. The lipid products of PI 3-K, the D3 phosphoinositides, could play a direct role in the formation of actin-containing motility structures, because the D3 phosphoinositides are known to bind to a number of proteins that regulate actin assembly, and to contribute to filopodial actin assembly in platelets.

Given our data, we next investigated the mechanism by which the α6β4 integrin activates PI 3-K. The preferential activation of PI 3-K by α6β4 compared to α6β1, as well as to other β1 integrins, in MDA-MB-435 cells suggests that the mechanism by which α6β4 activates PI 3-K differs either quantitatively or qualitatively from that of β1 integrins.

We know that the β4 cytoplasmic domain is required for PI 3-K activation, because a cytoplasmic domain mutant of the β4 subunit failed to increase PI 3-K activity upon ligation. The amino acid sequence of the β4 cytoplasmic domain is different from that of other integrin β subunits, yet it does not contain the consensus sequence for p85 binding via SH2 domains, YMXM, thus diminishing the possibility of a direct association with PI 3-K. In fact, we have not been able to detect such an association in our experiments. While we do not wish to bind ourselves to a specific mechanism, we believe the involvement of signaling intermediates is more likely.

One downstream effector of PI 3-K that we demonstrate is involved in carcinoma invasion is Rac, a small GTP-binding protein. Interestingly, neither constitutively active Rac, nor constitutively active Rho and Cdc42, were able to increase invasion of MDA-MB-435 cells. This suggests that activation of other PI 3-K-dependent pathways is also required in these cells. One downstream effector of Rac that we have determined is not involved in invasion is p70 S6K. Although p70 S6K is activated by $\alpha 6\beta 4$ ligation in the MDA-MB-435 cells, rapamycin, which blocks p70 S6K activation, did not inhibit invasion.

The PI 3-K signaling pathways involved in invasion appear distinct from those involved in PI 3-K-dependent cell survival. Even though the Akt kinase is activated by $\alpha 6\beta 4$ ligation, it is not required for invasion of the breast carcinoma cells that we studied. In fact, expression of a constitutively active form of Akt actually inhibited the invasiveness of these cells, most likely because activated Akt uses the D3-phosphoinositide products of PI 3-K at the expense of those PI 3-K-dependent pathways that are involved in invasion. A fascinating problem is raised by these observations because carcinoma progression involves both tumor cell invasion and survival. Akt is required for the survival of several cell types. This fact, coupled with our finding that PI 3-K is required for invasion, suggests that two essential functions of tumor progression may require the products of PI 3-K and that the balance between the use of these pathways may impact tumor cell invasion or survival.

In summary, we have identified a specific integrin-mediated pathway involving PI 3-K that promotes carcinoma invasion. This pathway involves the small GTP-binding protein Rac and protein kinase C. These findings are particularly important because they suggest that this PI 3-K signaling pathway is a target for inhibiting the spread of carcinomas with specificity.

Furthermore, activation of the PI 3-K signaling pathway may provide a means for promoting gastrointestinal wound healing.

The assays described herein can be used to test for compounds that modulate cell motility and/or invasiveness via PI 3-K, PKC, cAMP phosphodiesterase, or α6β4 integrin/actin interactions. Other members of the PI 3-K-dependent pathway that modulate cell motility and/or invasiveness also may be used to test for such compounds. Compounds identified using the methods provided herein may have therapeutic value in the treatment and prevention of cancer, particularly invasive carcinomas such as breast and colon carcinomas, and in the enhancement of wound healing.

Assays for the detection of compounds that modulate cell motility or invasiveness via a PI 3-K-dependent pathway

Potentially useful therapeutic compounds that modulate (e.g. increase or decrease) cell motility or invasiveness via PI 3-K, its upstream modulators, or its downstream effectors may be isolated by various screens that are well-known to those skilled in the art. Such examples include, but are not limited to, the Matrigel invasion assay and the phosphoinositide phosphorylation assay described in the Examples below. Useful compounds may, for example, modulate expression of PI 3-K or other molecules in the PI 3-K-dependent motility/invasiveness signaling pathway. Modulation of expression may be at the pre- or post-transcriptional level, or at the pre- or post-translational level.

Useful compounds also may modulate the biological activity of molecules in the PI 3-K-dependent pathway, e.g., by affecting post-translational modifications such as phosphorylation status. As shown in the Examples to follow, stimulation of PI 3-K biological activity enhances cell motility and/or invasiveness. This finding allows us to provide assays for drugs

that modulate cell motility and/or invasiveness by modulating PI 3-K biological activity. Such assays may measure, for example, PI 3-K biological activity by measuring changes in: (a) PI 3-K phosphorylation status; (b) PI 3-K association with cellular proteins; (c) levels of PI 3-K-induced cell motility or invasiveness; (d) levels of PI 3-K mRNA or gene expression, and (e) PI 3-K-induced lipid phophorylation. Analogous assays that measure changes in PKC or cAMP phosphodiesterase (cAMP-PDE) activity also may be used. Furthermore, assays that measure interactions between α6β4 integrin and actin may be employed. Such measurements may be made *in vitro* or *in vivo* and form the basis of assays which identify compounds that modulate cell motility and/or invasiveness. Such identified compounds may have therapeutic value in the treatment and/or prevention of cancer, particularly invasive cancers such as invasive breast or colon carcinomas. Other identified compounds, i.e., those that stimulate cell motility by activating PI 3-K, or PKC, etc., may have therapeutic value in enhancing wound healing, particularly by enhancing epithelial cell migration.

ELISA for the detection of compounds that modulate PI 3-K, PKC, or cAMP-PDE expression and/or activity

Enzyme-linked immunosorbant assays (ELISAs) are easily incorporated into high-throughput screens designed to test large numbers of compounds for their ability to modulate levels of a given protein. When used in the methods of the invention, changes in a given protein level of a sample, relative to a control, reflect changes in the PI 3-K (or Rac, Akt, PKC, or other downstream effectors of PI 3-K) expression status of the cells within the sample. Protocols for ELISA may be found, for example, in Ausubel et al.,*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1998. Lysates from cells treated with potential modulators of, for

example, PI 3-K expression are prepared (see, for example, Ausubel et al., *supra*), and are loaded onto the wells of microtiter plates coated with "capture" antibodies specific for PI 3-K. Unbound antigen is washed out, and a PI 3-K-specific antibody, coupled to an agent to allow for detection, is added. Agents allowing detection include alkaline phosphatase (which can be detected following addition of colorimetric substrates such as *p*-nitrophenolphosphate), horseradish peroxidase (which can be detected by chemiluminescent substrates such as ECL, commercially available from Amersham) or fluorescent compounds, such as FITC (which can be detected by fluorescence polarization or time-resolved fluorescence). The amount of antibody binding, and hence the level of a PI 3-K polypeptide within a lysate sample, is easily quantitated on a microtiter plate reader.

As a baseline control for PI 3-K expression, a sample that is not exposed to test compound is included. Housekeeping proteins are used as internal standards for absolute protein levels. A positive assay result, for example, identification of a compound that decreases PI 3-K expression, is indicated by a decrease in PI 3-K polypeptide within a sample, relative to the PI 3-K polypeptide level observed in cells which are not treated with a test compound.

Numerous variations of the basic assay may be employed. For example, in order to detect changes in PI 3-K phosphorylation status (and, hence, PI 3-K biological activity), duplicate ELISAS are used. The capture antibody for both assays is specific for PI 3-K, and the second (detection) antibody for the second assay is specific for phosphoprotein. Hence, the first ELISA gives a measure of the absolute quantity of PI 3-K present in a sample, whereas the second reveals the degree of PI 3-K phosphorylation in the sample. Duplicate ELISAs also are performed for the appropriate control samples. For example, in testing the effect of a compound on PI 3-K phosphorylation, the

duplicate ELISAs are also performed on control samples not treated with the test compound. Similar approaches may be used to isolate compounds that modulate interactions between α6β4 integrin and actin, e.g., by employing a capture antibody for β4 integrin, and a detection antibody for actin, or vice-versa.

Interaction trap assays

Two-hybrid methods, and modifications thereof, are used to screen for polypeptides that physically interact with PI 3-K, PKC or Rac, and hence might be members of the PI 3-K-dependent pathway that modulates cell motility and/or invasiveness. Regulators of PI 3-K, e.g. proteins that interfere with the interaction between PI 3-K and other proteins, also are identified by the use of a three-hybrid system. Similar approaches may be used to find proteins that regulate the interaction between actin and β4 integrin. Such assays are well-known to skilled artisans, and may be found, for example, in Ausubel et al., *supra*.

Reporter gene assays for compounds that modulate PI 3-K expression

Assays employing the detection of reporter gene products are extremely sensitive and readily amenable to automation, hence making them ideal for the design of high-throughput screens. Assays for reporter genes may employ, for example, colorimetric, chemiluminescent, or fluorometric detection of reporter gene products. Many varieties of plasmid and viral vectors containing reporter gene cassettes are easily obtained. Such vectors contain cassettes encoding reporter genes such as lacZ/β-galactosidase, green fluorescent protein, and luciferase, among others. Cloned DNA fragments encoding transcriptional control regions of interest (e.g. that of the PI 3-K gene)

are easily inserted, by DNA subcloning, into such reporter vectors, thereby placing a vector-encoded reporter gene under the transcriptional control of any gene promoter of interest. The transcriptional activity of a promoter operatively linked to a reporter gene can then be directly observed and quantitated as a function of reporter gene activity in a reporter gene assay.

Cells are transiently- or stably-transfected with PI 3-K control region/reporter gene constructs by methods that are well known to those skilled in the art. Transgenic mice containing PI 3-K control region/reporter gene constructs are used for late-stage screens *in vivo*. Cells containing PI 3-K/reporter gene constructs are exposed to compounds to be tested for their potential ability to modulate PI 3-K expression. At appropriate timepoints, cells are lysed and subjected to the appropriate reporter assays, for example, a colorimetric or chemiluminescent enzymatic assay for lacZ/β-galactosidase activity, or fluorescent detection of GFP. Changes in reporter gene activity of samples treated with test compounds, relative to reporter gene activity of appropriate control samples, indicate the presence of a compound that modulates PI 3-K expression. Analogous assays are used to screen for compounds that affect expression of other components of the PI 3-K-dependent pathway that regulates cell motility and/or invasiveness.

Quantitative PCR of PI 3-K mRNA as an assay for compounds that modulate PI 3-K expression

The polymerase chain reaction (PCR), when coupled to a preceding reverse transcription step (rtPCR), is a commonly used method for detecting vanishingly small quantities of a target mRNA. When performed within the linear range, with an appropriate internal control target (for example, the mRNA of a housekeeping gene such as actin), such quantitative PCR provides

an extremely precise and sensitive means of detecting slight modulations in mRNA levels.

Moreover, this assay is easily performed in a 96-well format, and hence is easily incorporated into a high-throughput screening assay. Cells are treated with test compounds for the appropriate time course, lysed, the mRNA is reverse-transcribed, and the PCR is performed according to commonly used methods, (such as those described in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1998), using oligonucleotide primers that specifically hybridize with PI 3-K nucleic acid. Such primers, the sequences of which will depend upon the specific PI 3-K (e.g., human, mouse, etc.) to be detected, are readily designed using well-known approaches such as software that can be used to design PCR primers. Changes in product levels of samples exposed to test compounds, relative to control samples, indicate test compounds that modulate PI 3-K expression.

Secondary screens of test compounds that appear to modulate cell motility and/or invasiveness via a PI 3-K-dependent pathway

After test compounds that appear to modulate cell motility or invasiveness via a PI 3-K-dependent pathway are identified, it may be necessary or desirable to subject these compounds to further testing. At late stages testing will be performed *in vivo* using animal models of invasive cancer or wound healing as appropriate, to confirm that the compounds initially identified to affect cell motility and/or invasiveness will have the predicted effect *in vivo*.

Test Compounds

In general, novel drugs (for the treatment and prevention of cancer, or for the enhancement of wound healing) that modulate the biological activity

of PI 3-K, its upstream activators, or its downstream effectors, are identified from large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials

already known for their therapeutic activities for the treatment and prevention of invasive cancer should be employed whenever possible.

When a crude extract is found to modulate cell motility and/or invasiveness via a PI 3-K-dependent signaling pathway, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract that modulates cell motility and/or invasiveness via a PI 3-K-dependent pathway. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed using mammalian models of invasive cancer (e.g., invasive breast or colon carcinoma) or wound healing.

Therapy

Compounds identified using any of the methods disclosed herein, may be administered to patients or experimental animals with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such compositions to patients or experimental animals. Although intravenous administration is preferred, any appropriate route of administration may be employed, for example, parenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal,

intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for antagonists or agonists of the invention include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

EXAMPLES

The following examples are to illustrate, not limit the invention.

EXAMPLE I: General Methods

Cells

The MDA-MB-435 breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository at Georgetown University. The MDA-MB-435 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Gibco) and 1%

penicillin-streptomycin (Gibco). Clone A cells were grown in RPMI supplemented with 25 mM Hepes (RPMI-H), 10% fetal calf serum and 1% penicillin-streptomycin.

Human T84 colon carcinoma cells were grown in DME-low glucose/Ham's F12 (GIBCO, Grand Island NY) supplemented with 6% normal calf serum, 2 mmol/L L-glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin. Cells were cultured for 2 to 3 days after reaching confluency before use in assays.

The cloning of the human β4 integrin cDNA, the construction of the β4 integrin cytoplasmic domain deletion mutant (β4-ΔCYT), and their insertions into the pRc/CMV (β4) and pcDNA3 (β4-ΔCYT) eukaryotic expression vectors respectively, have been described previously (Clarke et al., *J. Biol. Chem.* 270:22673-6, 1995). The vectors containing the full length and mutant β4 cDNAs, as well as the pcDNA3 vector alone, were transfected into the MDA-MB-435 cell line using Lipofectin (Gibco) according to the manufacturer's instructions. Neomycin resistant cells were isolated by selective growth in medium containing G418 (0.6 mg/ml; Gibco). The stable transfectants were pooled and populations of cells that expressed the human β4 subunit on the cell surface were isolated by FACS. A human β4 integrin-specific monoclonal antibody (mAb), UM-A9 (obtained from Tom Carey, University of Michigan, Ann Arbor, MI), was used for this sorting and for subsequent analysis of the transfectants. The sorting was repeated sequentially to enrich for homogeneous populations of cells expressing high levels of the transfected β4 and β4-ΔCYT subunits on the cell surface. Subclones were isolated from these populations by FACS. Surface labeling and immunoprecipitation with A9 were done to confirm that the α6β4 integrin heterodimer was expressed on these subclones.

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Analysis of Integrin Surface Expression

The relative surface expression of integrin subunits on the mock and β4-transfectants of the MDA-MB-435 cells was assessed by flow cytometry. For this purpose, aliquots of cells (5×10^5) were incubated for 45 minutes at room temperature with RPMI-H and 0.2% BSA (RH/BSA) and the following integrin-specific antibodies: 2B7 (anti-α6, etc.; prepared in our laboratory); mAb 13 (anti-β1; provided by Stephen Akiyama, National Institutes of Health, Bethesda, MD); A9 (anti-β4; provided by Thomas Carey, University of Michigan, Ann Arbor, MI), as well as mouse IgG (Sigma). The cells were washed two times with RH/BSA and then incubated with goat F(ab')2 anti-mouse IgG coupled to fluorescein (Tago) for 45 minutes at room temperature. After washing two times with RH/BSA, the cells were resuspended in the same buffer and analyzed by flow cytometry.

Invasion Assays

Matrigel invasion assays were performed as described (Shaw et al., *Cancer Res.* 56:959-63, 1996) using 6.5 mm Transwell chambers (8 μm pore size; Costar). Matrigel, purified from the EHS tumor, was diluted in cold distilled water, added to the Transwells (2-10 μg/well), and dried in a sterile hood. The Matrigel was then reconstituted with medium for an hour at 37°C before the addition of cells. Cells (5×10^5) were resuspended in serum-free medium containing 0.1% BSA and added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers. After 4-6 hours, the cells that had not invaded were removed from the upper face of the filters using cotton swabs and the cells that had invaded to the lower surface of the filters were fixed in methanol and then stained with a 0.2% solution of crystal violet in 2% ethanol. Invasion was quantitated by visual counting. The mean of five individual fields in the center of the filter where invasion was the highest was

obtained for each well. In some assays, the cells were pre-incubated for 30 minutes before addition to the Matrigel-coated wells with either wortmannin (Ui et al., *Trends in Biochemical Sciences* 20:303-7, 1995), PD98059 (Dudley et al., *Proc. Natl. Acad. Sci. USA* 92:7686-9. 1995; Pang et al., *J. Biol. Chem.* 270:13585-8, 1995), or rapamycin (Chung et al., NEED REF 1992; Kuo et al., *Nature* 358:70-3, 1992; Price et al., *Science* 257:973-7, 1992). For assays that used the transient transfectants described below, the cells were fixed for 30 minutes in 4% paraformaldehyde and then stained with PBS containing 1 mg/ml Bluo-gal (Boehringer Mannheim), 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide.

Kinase Assays

Cells were removed from their dishes with trypsin and washed twice with RPMI-H containing 0.2% heat-inactivated BSA. After washing, the cells were resuspended in the same buffer at a concentration of 2 x 10^6 cells/ml and incubated for 30 minutes with integrin-specific antibodies or in buffer alone. The cells were washed once, resuspended in the same buffer and added to plates that had been coated overnight with either anti-mouse IgG or laminin-1. After a 30 minute incubation at 37°C, the cells were washed twice with cold PBS and solubilized at 4°C for 10 minutes in a 20 mM Tris buffer, pH 7.4, containing 0.14 M NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonylfluoride (PMSF), 5 μg/ml aprotinin, pepstatin and leupeptin. Nuclei were removed by centrifugation at 12,000 x g for 10 minutes.

To assay PI 3-K activity, aliquots of cell extracts that contained equivalent amounts of protein were incubated for 3 hours at 4°C with either the anti-phosphotyrosine mAb 4G10 (UBI) or a p85 subunit-specific PI 3-K antibody and Protein A sepharose (Pharmacia). The sepharose beads were

washed twice with solubilization buffer and twice with a 10 mM Hepes buffer, pH 7, containing 0.1 mM EGTA (kinase buffer). After removal of the last wash, the beads were resuspended in kinase buffer containing 10 μg of sonicated crude brain lipids (Sigma), 100 μM ATP, 25 mM $MgCl_2$, and 10 μCi g-^{32}P-ATP and incubated for 10 minutes at room temperature. The reaction was stopped by the addition of 60 μl 2 N HCl and 160 μl of a 1:1 mixture of chloroform and methanol. Lipids were resolved by thin layer chromatography plates coated with potassium oxalate.

To assay MAP kinase activity, total cell extracts, prepared as described above, were resolved by electrophoresis on SDS-polyacrylamide gels (10%), transferred to nitrocellulose, and blotted with a phospho-specific ERK polyclonal antibody that recognizes the phosphorylated isoforms of ERK-1 and ERK-2 (New England BioLabs, Inc.). Immune complexes were detected using a secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (Amersham, Inc.). The blots were then stripped and re-probed with an ERK-1 antibody (provided by John Blenis, Harvard Medical School, Boston, MA) that recognizes both ERK-1 and ERK-2.

To assay Akt kinase activity, total cell extracts containing equivalent amounts of protein were pre-cleared with a 1:1 mixture of Protein A/Protein G and then incubated with a polyclonal antibody that recognizes the carboxy-terminal end of Akt (provided by Thomas Franke, WHERE FROM) for 3 hours at 4°C. After a 1 hour incubation with the Protein A/Protein G mixture, the beads were washed 3 times with solubilization buffer, once with H_20, and once with a 20 mM Hepes buffer pH 7.4 containing 10 mM $MgCl_2$ and 10 mM $MnCl_2$ (kinase buffer). After removal of the last wash, the beads were resuspended in 30 μl of kinase buffer containing 5 μM ATP, 1 mM DTT, 10 μCi g-^{32}P-ATP, and 1.5 μg of Histone H2B (Boehringer Mannheim) and incubated for 20 minutes at room temperature. The reaction was stopped by the

addition of 5X Laemmli sample buffer and resolved by electrophoresis on SDS-polyacrylamide gels (12%).

To assay protein kinase C (PKC) activity, experimental conditions were the same as for Akt kinase assays except myelin basic protein is used as a substrate for phosphorylation by PKC.

To assay p70 S6K activation, total cell extracts, prepared as described above, were resolved by electrophoresis on SDS-polyacrylamide gels (8%), transferred to nitrocellulose, and blotted with a polyclonal antibody that recognizes the C-terminal end of p70 S6K (provided by John Blenis, Harvard Medical School, Boston, MA). Immune complexes were detected using a secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (Amersham, Inc.).

Transient Transfections

The constitutively active PI 3-K catalytic p110 subunit was a generous gift of Julian Downward, ICRF, London. The small GTP-binding proteins V14Rho, V12Rac, V12Cdc42, N17Rac and N17Cdc42, in the pEBG vector, were a kind gift of Margaret Chou, University of Pennsylvania, Philadelphia, PA. The constitutively active Akt was kindly provided by Philip Tsichlis, Fox Chase Cancer Center, PA. The dominant-negative PI 3-K Dp85 subunit was kindly provided by Brian Schaffhausen, Tufts University. The pCS2-(n)β-Gal was a gift from Sergei Sokol, Beth Israel Deaconess Medical Center.

Cells were co-transfected with 1 μg pCS2-(n)β-Gal and the specified cDNAs using Lipofectamine (Gibco) according to the manufacturer's instructions. Cells were harvested 24 hours after transfection and added to Matrigel invasion assays as described. Transfected cells were also plated in 48-well plates to stain for β-galactosidase expression to determine transfection

efficiency. The remaining cells were collected and extracted in RIPA buffer (phosphate buffered saline, pH 7.4, containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, and 5 μg/ml aprotinin, leupeptin, and pepstatin). These cell extracts were immunoprecipitated with HA- (Myr-Akt; Boehringer Mannheim), Myc- (Myr-p110 and p85; Oncogene Science), or GST- (V14Rho, V12Rac, V12Cdc42, N17Rac, N17Cdc42 and Dp85; Santa Cruz) specific antibodies, resolved by electrophoresis on SDS-polyacrylamide gels (10%), and transferred to nitrocellulose. The expression of the tagged proteins was detected by immunoblotting with the same antibodies.

cAMP Assays

Culture dishes (35 mm) were coated overnight with 20μg/ml of either laminin-1 or collagen I in PBS and then blocked with serum-free RPMI containing 250 μg/ml BSA (RPMI/BSA). Cells (1.5×10^6) were then plated for 2 hrs on these dishes and harvested by quickly removing the medium and extracting the cells directly with 80% (v/v) ethanol. Cell extracts were collected, cleared by centrifuging in a microcentrifuge at full speed for 10 minutes, dried in a SpeedVac for 1.5 hrs., and resuspended in 50 mM phosphate buffer, pH 6.2. The intracellular cAMP concentration was quantified using a cAMP enzyme-linked immunoabsorption assay (cAMP EIA; Cayman Biochemicals, Ann Arbor, MI) following the manufacturer's instructions, using non-acetylated cAMP as a standard and acetylcholine esterase-linked cAMP as a competitor. Values were corrected for cell number as determined from replicate plates. In some experiments, 50μM forskolin, 12 mM IBMX or 100 nM LPA was added to cells 15 minutes prior to harvesting. For determination of cAMP content of cells under normal culture conditions, cells were plated in 35mm dishes in DMEM plus 10% FCS, incubated for 18 hrs and processed as described above. The effect of integrin ligation on cAMP

levels was determined by treating cells in suspension with 4μg/ml antibody for 30 min. and then seeding them on plates (35mm) coated with 50μg goat anti-mouse IgG antibody for 15 min. prior to harvesting for analysis of cAMP levels.

Phosphodiesterase Assays

cAMP phosphodiesterase (PDE) assays were performed according to the protocol of Sette et al., *J. Biol. Chem.* 269:9245-9252, 1994). Briefly, cells were harvested in PDE lysis buffer (20 mM Tris-HC1, pH 8.0, 1 mM EDTA, 0.2 mM EGTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5μg/ml leupeptin, 0.7μg/ml pepstatin, 4μg/ml aprotinin, and 2 mM PMSF) and sonicated. Cellular debris was removed by centrifugation and the supernatants were assayed immediately for PDE activity. PDE activity of cell extracts (2-4μg protein) was assayed in cAMP-PDE assay buffer (20 mM Tris-HC1, pH 8.0, 10 mM $MgCl_2$, 1.25 mM β-mercaptoethanol, 0.14 mg/ml BSA, 1μM cAMP and 0.2 μCi [^{3}H]-(cAMP) for 10 min. at 34°C. The reaction was stopped by adding 40 mM Tris, pH 7.5, containing 10 mM EDTA and heating for 2 min. at 100°C. The reaction products were then digested with 50μg *Crotalus atrox* snake venom (Sigma) for 30 min. at 34°C, separated from substrate using SpinZyme Acidic Alumina Devices (Pierce Biochemical), and quantified using a scintillation counter. Values were corrected for protein content and are reported as pmol cAMP hydrolyzed per min. per mg protein. Protein content of cell extracts was determined using the BioRad Protein Reagent with BSA as a protein standard.

Immunofluorescence Microscopy

T84 cells were dissociated with trypsin/EDTA and plated onto the permeable supports (8-μm pore size) of Transwell chambers (Costar,

Cambridge, MA). Growth medium was placed in the upper and lower chambers and the cells were grown in a 37°C, 5% CO_2 atmosphere until a confluent monolayer was formed. Subsequently, the monolayers were wounded by aspiration through a flame-polished pipette tip. Before immunostaining, some of the samples were treated for 15 minutes with 2 mmol/L EDTA in calcium- and magnesium-free phosphate-buffered saline (PBS) to ensure antibody access to the basal regions of the monolayers. In some experiments, the cells were treated with 1% Triton X-100 in PBS (pH 7.4) for 10 minutes before fixation with paraformaldehyde (4% in PBS for 10 minutes). After fixation, the permeable supports were cut from the Transwell chambers and incubated in a blocking solution (3% bovine serum albumin and 1% normal donkey serum in PBS) for 1 hour at room temperature. After incubation with either primary antibody, nonspecific IgG, or nonspecific ascites fluid, the permeable supports were washed in PBS (three times, 10 minutes each) and incubated for 1 hour at room temperature in fluorescein-conjugated goat anti-mouse IgG (1:100). All antibodies were diluted in blocking solution. After staining, the permeable supports were washed in PBS and mounted in a mixture (8:2) of glycerol and PBS, pH 8.5, containing 1% propylgallate. Slides were examined by confocal imaging (MRC 600, BioRad Microsciences, Cambridge, MA) using a Zeiss Axiovert inverted microscope (Carl Zeiss, Thornwood, NY).

Cells and antibodies for α6β4 integrin-actin co-localization studies

The clone A cell line was originally isolated from a human, poorly differentiated colon adenocarcinoma (Dexter, D.L., et al., *Cancer Res.* 39:1020-1025, 1979), and its *in vitro* properties and repertoire of integrin receptors have been described previously (Daneker, G.W., et al., *Cancer Res.* 49:681-686, 1989; Lee, E.C., et al., *J. Cell Biol.* 117:671-678, 1992; Lotz, M.M., et al., *Cell*

BNSDOCID: <WO 9935283A1 >

Regul. 1:249-257, 1990; and Tozeren, A., et al., *J. Cell Sci.* 107:3153-3163, 1994). The CCL-228 colon carcinoma and the MDA-231 breast carcinoma cell lines were obtained from American Type Culture collection (Rockville, MD). The MIP-101 colon carcinoma cell line has been described previously (Deneker, G.W., et al., *Cancer Res.* 49:681-686, 1989). Cells were grown in RPMI 1640 medium containing 10 mM Hepes, penicillin (50 U/μl), streptomycin (50 μg/ml), and 10% FBS.

The following mAbs were used in this study: mouse mAb 287 (anti-integrin α6) prepared in our laboratory; rat mAB GoH3 (anti-integrin α6) from Immunotech (Westbrook, ME); mouse mAb K20 (anti-integrin β1) provided by Steven Akiyama (National Institutes of Health, Bethesda MD) (Akiyama, S.K., et al., *J. Cell Biol.* 102:442-448, 1986); mouse mAb A9 (anti-integrin β4) provided by Thomas Carey (University of Michigan, Ann Arbor, MI); and mouse anti-pan-cytokeratin (a mixture of antibodies that recognizes cytokeratins 1, 4, 5, 6, 8, 10, 13, and 19) from Sigma Chemical Co. (St. Louis, MO).

Cell Migration Assays

To assay the migration of clone A cells, bacteriological dishes were coated with 10-100 μg of laminin-1 prepared from the EHS sarcoma as described (27 NEED REF) or collagen type I (Collaborative Research, Waltham, MA) for 2 h. at room temperature and then blocked with PB5 containing 1% BSA for 1 h. Clone A cells in exponential growth were removed from culture dishes and resuspended in serum-free RPMI 1640 medium containing 10 mM Hepes and 0.1% BSA. The cells were then plated at low density (1 X 10^4/cm^2) on the matrix-coated dishes and allowed to adhere for 30 min. in a humidified atmosphere with 5% CO_2 at 35°C. In some experiments, integrin-specific antibodies (2B7 or MC-13, 10 μg/ml) were added to the cells

either before the cells were plated or after the cells had adhered for the 30 min.. The dishes were then sealed with parafilm and placed on a microscope stage heated to 37°C. For image analysis, an inverted microscope (model Diaphol 300: Nikon, Inc., Melville, NY) with phase contrast optics was used. This microscope was connected to a CCD camera (Dage-MTI, Michigan City, IN), a frame-grabber (Sclon), and a 7600 Power Macintosh computer (Cupertino, CA) to capture the images. Images were collected for 1 h. and analyzed with Iplab Spectrum image analysis software.

Migration speed was determined by following cell centroid displacements as a function of time for 1 h. at intervals of 15 min. For each individual experiment, 30-40 cells were analyzed. A frame-by-frame analysis of filopodia at intervals of 1 min. over the course of 1 h. was used to differentiate filopodia from retraction fibers and to monitor the formation and stabilization of filopodia. Lamellar area was determined by tracing lamellar contour and quantifying the area digitally.

Indirect Immunofluorescence Microscopy

Clone A cells were plated on matrix-coated dishes as described above and incubated for 1 h. in a humidified atmosphere with 5% CO_2 at 37°C. The cells were then fixed for 20 min. at room temperature with a buffer containing 4% paraformaldehyde, 100 mM KCl , 300 mM sucrose, 2 mM EGTA, 2 mM $MgCl_2$, and 10 mM Pipes at pH 6.8. The cells in some experiments were extracted for 1 min. at 4°C before fixation with either a "membrane" buffer containing 0.5% Triton X-100, 100 mM KCl, 300 mM sucrose, 10 mM EGTA, 2 mM $MgCl_2$, 1 mM PMSF, and 10 mM Pipes at pH 6.8 or for 5 min. at room temperature with a "cytoskeletal" buffer containing 1% Tween-40, 0.5% sodium deoxycholate, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF, and 20 mM tris-HCl, pH 7.4, and then fixed for 20 min. in the

paraformaldehyde buffer. Subsequently, the fixed cells were rinsed with PBS and incubated with a blocking solution that contained 1% albumin and 5% donkey serum in PBS for 30 min. Primary antibodies (GoH3, 1:50: K20, 1:50; pan-cytokeratin, 1:200) and/or FITC phalloidin (20 μg/ml) in blocking solution were incubated with the cells separately or in combination for 30 min. The cells were rinsed three times and either a fluorescein-conjugated donkey anti-mouse or a rhodamine-conjugated donkey anti-rat IgG (minimal cross-reactions interspecies, Jackson ImmunoResearch Labs, West Grove, PA) in blocking buffer (1:150) were used separately or in combination to stain the cells for 30 min. Cells were rinsed with PBS and mounted in a mixture (8:2) of glycerol and PBS, pH 8.5, containing 1% propylgallate. The dishes were cut into slides and examined by confocal microscopy (model LSM; Carl Zeiss, Inc., Thorwood, NY).

Actin-severing Experiments

Clone A cells (2 x 10^6) suspended in RPMI-H with 0.1% albumin were plated on laminin-1-coated dishes and incubated for 1 h. at 37°C. The following steps were done at 4°C. The medium was removed and a membrane buffer (see above) was added for 30 s. and removed by aspiration. A "low calcium" buffer (25 μm $CaCl_2$, 100 mM KCl, 300 mM sucrose, 10 mM EGTA, 2 mM $MgCl_2$, leupeptin (10 μg/ml), aprotinin (1 μg/ml), pepstatin (5 μg/ml), and 10 mM Pipes, pH 6.8) was used to remove the membrane buffer by washing the cells four times with gentle rocking. Subsequently, the low calcium buffer containing 200 nM gelsolin (kindly provided by Dr. Paul Janmey) and 50 μg/ml of GC-globulin (Calbiochem, La Jolla, CA) was added to the cells and incubated for 30 min. Control cells were treated with the low calcium buffer alone. An equal volume of membrane buffer was added to the cells for 30 s. to terminate the reaction. The buffer was removed and collected

in microfuge tubes, cetrifuged at 12,000 rpm for 10 min. and immunoprecipitated with the 287 (anti-α6) antibody. The immune complexes were resolved by SDS-PAGE and immunoblotted with an anti-β4 integrin polyclonal antibody elicited against the last 20 amino acids of the β4 cytoplasmic tail.

EXAMPLE II: Expression of the α6β4 integrin increases the invasiveness of MDA-MB-435 breast carcinoma cells.

The MDA-MB-435 cells used in this study do not express the α6β4 integrin although they express the wild-type α6β1 integrin. Stable subclones of these cells were generated that express either the α6β4 integrin or a mutated α6β4 integrin that lacks the β4 cytoplasmic domain, with the exception of the four amino acids proximal to the transmembrane domain (β4-ΔCYT). Subclones of transfected MDA-MB-435 cells expressing β4 on the cell surface were isolated by FACS using UM-A9, a mAb specific for the β4 integrin subunit. MDA-MB-435 cells transfected with vector alone (subclones 6D2 and 6D7) or the human β4 integrin subunit (subclones 3A7 and 5B3) were analyzed by flow cytometry using monoclonal antibodies specific for the indicated integrin subunits. The relative surface expression of the α6, β4 and β1 subunits on the subclones used in this study is shown in Fig. 1.

Expression of the β4 subunit did not alter surface expression of the α6 subunit (Fig. 1) or other integrin α subunits on these cells. However, a slight decrease in β1 surface expression was observed in the β4 transfectants that probably reflects a decrease in α6β1 expression in response to α6β4 expression (Fig. 1).

The possibility that expression of the α6β4 integrin stimulates the invasion of carcinoma cells was examined by comparing the ability of mock transfectants (6D2 and 6D7) and β4 transfectants (3A7 and 5B3) to invade

Matrigel in a standard chemoinvasion assay (Albini et al., *Cancer Res.* 47:3239-45, 1987). In the experiment depicted in Fig. 2A, "Mock" indicates MDA-MB-435 cells transfected with vector alone; "β4" indicates MDA-MB-435 cells transfected with the full length β4 subunit; and "β4-ΔCYT" indicates MDA-MB-435 cells transfected with the mutant β4 subunit lacking the cytoplasmic domain. The data shown are from two individual subclones of each transfectant and are the mean values (+SD) of a representative experiment done in duplicate.

As shown in Fig. 2A, the rate of invasion of the β4 transfectants was approximately 3-4 fold greater than that of the mock transfectants in a 4 hour assay. The β4-ΔCYT transfectants invaded at a slightly slower rate than that of the mock transfectants (Fig. 2A) indicating that the β4 cytoplasmic domain is essential for stimulating invasion. Interestingly, the rate of adhesion to laminin was not greater in the β4 transfectants than in the mock transfectants.

EXAMPLE III: Antibodies specific for the α6β4 integrin stimulate invasion of MDA-MB-435 breast carcinoma cells.

To examine the contribution of integrin receptors to the invasion of MDA-MB-435 cells, Matrigel chemoinvasion assays were performed in the presence of integrin subunit-specific antibodies. In the experiment depicted in Fig. 2B, cells were pre-incubated for 30 minutes in the presence of antibodies before addition to the Matrigel-coated wells. After 4 hours at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in experimental procedures. "IgG" indicates non-specific antibody; "β1" indicates the anti-β1 mAb 13; and "α6" indicates the anti-α6 mAb 2B7. The data shown are from two individual subclones of each transfectant and are the mean values (+SEM) of a representative experiment done in triplicate.

As shown in Fig. 2B, the β1-specific antibody mAb 13 inhibited invasion of the mock and β4-transfectants. The α6-specific mAb 2B7 inhibited invasion of the mock transfectants by approximately 60%, in agreement with our previous result that these cells use α6β1 as a major laminin receptor. However, the same antibody increased the rate of invasion of the β4 transfectants by approximately 30%. The stimulation of invasion observed for the α6 antibody in the MDA-MB-435/β4 transfectants suggests that α6β4 is not required for the adhesive functions involved in invasion but rather acts as a signaling receptor whose function can be enhanced by antibody binding. This possibility is also supported by the finding mentioned above that α6β4 expression did not increase the rate of adhesion of MDA-MB-435 cells to laminin-1. These data indicate that the adhesive functions of the MDA-MB-435/β4 transfectants required for invasion are mediated largely by β1 integrins.

EXAMPLE IV: Invasion of MDA-MB-435 breast carcinoma cells is dependent on PI 3-K.

In initial experiments toward elycidating the signaling mechanism by which the α6β4 integrin stimulates invasion, MDA-MB-435 transfectants were assayed for their ability to invade Matrigel in the presence of either the MEK inhibitor PD98059 (25 μM), the PI 3-K inhibitor wortmannin (100 μM), or the p70 S6K inhibitor rapamycin (20 ng/ml). Cells were preincubated for 10 minutes in the presence of the inhibitors before addition to the Matrigel-coated wells. After 4 hours at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in experimental procedures. The data shown are the mean values (+SD) of a representative experiment done in duplicate.

"Mock" indicates MDA-MB-435 cells transfected with vector alone, and "β4" indicates MDA-MB-435 cells transfected with full length β4 subunit.

We assessed first the effects of the MAPK kinase inhibitor PD98059 on MDA-MB-435 invasion (Dudley et al., 1995; Pang et al., 1995). As shown in Fig. 3A, pre-treatment of these cells with PD98059 (25 μM) resulted in only a modest inhibition (20%) of invasion.

To confirm that PD98059 inhibits MAPK activity in these cells, an antibody that recognizes the phosphorylated, active isoforms of ERK1 and ERK2 was used to probe extracts of PD98059-treated cells. MDA-MB-435 transfectants were maintained in suspension (SUS) or incubated with α6-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes in the absence or presence of PD98059 or wortmannin (WT). Aliquots of total cell extracts were normalized for protein content and resolved by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and immunoblotted using an ERK polyclonal antibody that recognizes the phosphorylated isoforms of ERK-1 and ERK-2 (p-ERK-1 and p-ERK-2; Fig. 3B, upper bands). The blots were then stripped and re-probed with an ERK-1 antibody that recognizes both ERK-1 and ERK-2 to confirm that the total amount of ERK-1 and ERK-2 protein was not altered by either PD98059 or WT (Fig. 3B, lower bands). Antibody-induced clustering of the α6 integrins in both the mock and β4 transfectants using 2B7, an α6-specific antibody, stimulated activation of MAPK as assessed by reactivity with the phospho-specific MAPK antibody (Fig. 3B). This activation was inhibited by PD98059 (25 μM). Of note, α6β4 expression did not have a significant impact on α6-induced MAPK activation in these cells.

We next targeted PI 3-K as a mediator of invasion because of its central involvement in multiple signaling pathways. The PI 3-K inhibitor wortmannin (WT) inhibited invasion of both the mock and β4 transfectants by

70-80% (Fig. 3A). In contrast to PD98059, WT did not inhibit activation of MAPK by antibody-induced clustering of the α6 integrins in either the mock or β4 transfectants (Fig. 3B). Taken together, these results suggest that PI 3-K, but not MAPK, is necessary for the invasion of MDA-MB-435 cells.

EXAMPLE V: Activation of PI 3-K by the α6β4 integrin.

To determine whether α6β4 integrin could stimulate PI 3-K activity, *in vitro* kinase assays were performed using the mock (α6β1) and β4 (α6β4), and β4-ΔCYT transfectants of MDA-MB-435 breast carcinoma cells (Fig. 4A). MDA-MB-435 transfectants were maintained in suspension or incubated with integrin-specific antibodies and allowed to adhere to anti-mouse IgG-coated plates or laminin-1 coated plates for 30 minutes. "SUS" indicates cells maintained in suspension, and "α6" indicates cells clustered with the α6-specific antibody. After ligation of the α6 integrins with antibody 2B7, aliquots of cell extracts that contained equivalent amounts of protein were immunoprecipitated by incubating with the phosphotyrosine-specific monoclonal antibody 4G10 and Protein A Sepharose for 3 hours to capture the activated population of PI 3-K. After washing, the beads were resuspended in kinase buffer and incubated for 10 minutes at room temperature and the immunoprecipitates were assayed for their ability to phosphorylate crude brain phosphoinositides. Phosphorylated lipids were resolved by thin layer chromatography.

As shown in Fig. 4A, an increase in PI 3-K activity, indicated by the appearance of PtdIns-3,4,5-P3, was observed upon clustering the α6β1 integrin in the mock transfectants and the α6β4 integrin in the β4 transfectants. More importantly, PI 3-K activity stimulated by clustering the α6β4 integrin was markedly greater than that observed after clustering the α6β1 receptor. This

enhanced stimulation of PI 3-K was also seen using a β4-specific mAb to ligate the α6β4 integrin in the β4 transfectants (Fig. 4B).

PI 3-K activity was higher in the β4 transfectants (α6β4) than in the mock transfectants (α6β1) after adhesion to laminin-1 (Fig. 4A). This observation suggests that interactions with laminin through this receptor can stimulate PI 3-K activity even though α6β4 is not used as an adhesion receptor in these cells. PI 3-K activity was not increased upon ligation of the α6β4-ΔCYT receptor (Fig. 4B) and little PI 3-K activity was evident when the transfectants were maintained in suspension (Fig. 4A).

Our data suggested that the ability of the α6β4 integrin to activate PI 3-K may be quantitatively greater than that of β1 integrins in MDA-MB-435 cells. This possibility was examined by comparing PI 3-K activation in the β4 transfectants in response to antibody ligation of either β1 integrins or the α6β4-integrin. MDA-MB-435 cells were transfected with the full length β4 subunit (cell clones 3A7 and 5B3), or the β4 subunit lacking the cytoplasmic domain (β4-ΔCYT), and the cells were clustered with either the β1-specific antibody or the β4-specific antibody. The samples were then tested for PI 3-K activation, and the integrin-activated levels of PtdIns-3,4,5-P3 generated by laminin-adherent transfectants were compared to the levels observed for transfectants that were maintained in suspension; the value from this ratio was determined to be the relative PI 3-K activity stimulated by each integrin subunit.

Fig. 4B shows a densitometric analysis of the amount of radiolabeled PtdIns-3,4,5-P3 generated by the antibody-treated β4 (3A7 and 5B3) and β4-ΔCYT integrin transfectants. The data shown are the mean values (+SD) from two representative experiments. The results shown in Fig. 4B indicate that ligation of the α6β4 integrin with β4-specific antibodies stimulated PI 3-K activity approximately two-fold more than did β1 integrin ligation,

demonstrating that PI 3-K is activated preferentially by the α6β4 integrin. The differences between the abilities of the α6β4 and β1 integrins to activate PI 3-K are most likely even greater than what was observed, given the two-three fold higher level of expression of β1 than β4 integrins on the cell surface (Fig. 1).

EXAMPLE VI: Constitutively active PI-3K stimulates invasion of MDA-MB-435 breast carcinoma cells.

The hypothesis that the α6β4 integrin promotes invasion of MDA-MB-435 cells by enhancing the activity of PI 3-K implies that expression of a constitutively active form of PI 3-K in the parental cells should increase their invasion in the absence of α6β4 expression. To validate this prediction, a constitutively active, membrane-targeted PI 3-K was expressed transiently in the parental MDA-MB-435 cells and the ability of these cells to invade Matrigel was compared to cells transfected with an empty vector (Fig. 5A). Specifically, MDA-MB-435 cells were transiently transfected with 1 μg pCS2-(n)β-gal as an internal control for transfection efficiency and 4 μg of either the vector alone or a Myc-tagged, constitutively active form of the PI 3-K p110 catalytic subunit (Myr-p110) and assayed for their ability to invade Matrigel in the absence or presence of wortmannin (100 nM). Expression of the transiently expressed p110 subunit was confirmed by immunoblotting using a myc-specific antibody. The data shown are the mean values (+SD) of two (wortmannin) or three (without wortmannin) experiments done in triplicate. As shown in Fig. 5A, constitutively active PI 3-K increased invasion two-fold, and this invasion was inhibited by wortmannin.

EXAMPLE VII: α6β4-dependent invasion requires PI-3K activity.

If the α6β4 integrin promotes invasion of MDA-MB-435 cells by enhancing the activity of PI 3-K, expression of a dominant-negative PI 3-K subunit in the β4 transfectants should decrease their invasion. MDA-MB-435/β4 transfectants were transiently transfected with 1 μg pCS2-(n)β-gal and either the vector alone, 6 μg of a PI 3-K p85 subunit deleted in the p110 binding site (Δp85), or 6 μg of a wild-type PI 3-K p85 regulatory subunit (p85) and assayed for their ability to invade Matrigel (Fig. 5B). After 5 hours at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained. Invasion was quantitated by counting the cells that stained positively for β-galactosidase expression. Relative invasion was determined by comparing the amount of invasion obtained for the experimental transfections to that observed for the cells transfected with the vector alone, which was given the value of 1. The expression of the transfected cDNAs was confirmed by immunoblotting and is shown below each graph. The data shown are the mean values (+SD) of two experiments done in triplicate (Fig. 5B).

Transient expression of a GST-tagged, PI 3-K p85 subunit deleted in the p110 binding site (Δp85) inhibited invasion of the MDA-MB-435/β4 transfectants significantly (Fig. 5B). A similar inhibition of invasion was observed after transient expression of a wild type p85 subunit. It has been shown that overexpression of the wild type p85 subunit blocks PI 3-K activation by binding to phosphotyrosine-containing proteins and inhibiting the binding of endogenous p85/p110 to these proteins (Rameh et al., *Cell* 83, 821-30, 1995). The data obtained with the Δp85 and wild-type p85 subunits substantiate the wortmannin data shown in Fig. 3A and confirm the involvement of PI3-K signaling in α6β4-dependent invasion.

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EXAMPLE VIII: The Akt/PKB kinase and p70 S6 kinase, downstream effectors of PI-3K, are not required for invasion.

Because the Akt/PKB serine/threonine kinase (Akt) and the p70 S6 kinase (S6K) are activated downstream of PI 3-K, we hypothesized that these kinases could play important roles in invasion.

To assess the potential role of Akt in invasion, MDA-MB-435/β4 transfectants were maintained in suspension or incubated with α6-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes. Cell extracts made from these samples were incubated with a polyclonal anti-Akt antibody and a 1:1 mixture of Protein A/Protein G, and the bead-coupled protein was subject to a kinase assay. The phosphorylated substrate, histone H2B, is shown in Fig. 6A.

To assess the potential role of S6K in invasion, aliquots of extracts from cells treated as described above were normalized for protein content, resolved by 8% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and immunoblotted using a polyclonal antibody that recognizes the C-terminal end of p70 S6K (Fig. 6A). As shown in Fig. 6A, ligation of the α6β4 integrin in the MDA-MB-435/β4 transfectants activated both Akt and S6K, enhancing the hypothesis that Akt and S6K play important roles in invasion.

Based on the observation that Akt and S6K kinase activity were activated by ligation of α6β4 integrin, we next examined the ability of parental MDA-MB-435 cells that expressed a constitutively active form of Akt (Myr-Akt) to invade Matrigel. Surprisingly, the constitutively active form of Akt actually decreased the rate of invasion in comparison to the control cells, even though it was expressed at relatively high levels (Fig. 6B). Most likely, the exogenously expressed active Akt sequestered a significant fraction of D3

phosphoinositides and precluded the use of these lipids in those signaling pathways downstream of PI 3-K that are required for invasion.

We examined the involvement of S6K in invasion using rapamycin, a specific inhibitor of S6K activation (Chung et al., *Cell* 69:1227-36, 1992; Kuo et al., *Nature* 358:70-3, 1992; Price et al., *Science* 257:973-7, 1992). As shown in Fig. 3, rapamycin did not decrease the invasion of either the mock or β4 transfectants. Based on these results, we conclude that Akt and S6K are not required for MDA-MB-435 invasion.

EXAMPLE IX: The small G-protein Rac is required for MDA-MB-435 invasion.

The Rho family of small G-proteins is involved in the actin rearrangements that result in the formation of stress fibers, membrane ruffles and lamellae, and filopodia (Nobes and Hall, *Cell* 81:53-62, 1995). The ability of cells to form these actin-containing structures is linked to their motility and therefore could influence their invasive potential (Rabinovitz and Mercurio, Journal of Cell Biology (In Press), 1997; Sheetz, *Seminars in Cell Biology* 5:149-55, 1994). To examine this possibility, constitutively active mutants of either Rho (V14Rho), Rac (V12Rac), or Cdc42 (V12Cdc42) were transiently expressed in the parental MDA-MB-435 cells. MDA-MB-435 cells were transiently transfected with 1 μg of pCS2-(n)β-gal and either 4 μg of the vector alone, or 4 μg of constitutively active mutants of Akt (Myr-Akt), Rac (V12Rac), Cdc42 (V12Cdc42), or Rho (V14Rho) and assayed for their ability to invade Matrigel. After 5 hours at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained. Invasion was quantitated by counting the cells that stained positive for β-galactosidase expression. Relative invasion was determined by comparing the amount of invasion obtained for the experimental transfections

to that observed for the cells transfected with the vector alone, which was given the value of 1. The data shown are the mean values (+SD) of 3 experiments done in triplicate. As shown in Figure 6B, independent expression of these constitutively active small G-proteins did not significantly alter the invasion of MDA-MB-435 cells indicating that they are not sufficient by themselves to increase invasion.

To determine if either Rac or Cdc42 contributed to α6β4-dependent invasion, MDA-MB-435/β4 transfectants were transiently transfected with 1 μg of pCS2-(n)β-gal and either 4 μg of vector alone, or 4 μg of dominant-negative mutants of Rac (GST-N17Rac) or Cdc42 (N17Cdc42) and assayed for their ability to invade Matrigel as described above. The data shown are the mean values (+SD) of a representative experiment done in triplicate.. A significant reduction (50%) in invasion was observed when dominant-negative N17Rac was transiently expressed in the MDA-MB-435/β4 transfectants. In contrast, expression of dominant-negative N17Cdc42 did not inhibit invasion significantly (Fig. 6C).

To examine the role of Rac in PI 3-K stimulated invasion of parental MDA-MB-435 cells, dominant-negative N17Rac was transiently expressed along with the constitutively active form of the PI 3-K p110 catalytic subunit (Fig. 6D). Specifically, MDA-MB-435 cells were transiently transfected with 1 μg of pCS2-(n)β-gal and either 5 μg of the vector alone, 3 μg of the vector alone and 2 μg of a Myc-tagged constitutively active PI 3-K p110 subunit (Myr-p110) or 2 μg of Myr-p110 and 3 μg N17Rac, and assayed for their ability to invade Matrigel as described above. The data shown are the mean values (+SD) of a representative experiment done in triplicate. The expression of the transfected cDNAs was confirmed by western blotting and is shown below each graph.

As shown in Fig. 6D, co-expression of dominant-negative N17Rac inhibited the increased invasion that was observed when the constitutively active p110 subunit of PI 3-K was expressed alone. Taken together, these results demonstrate that Rac is an essential downstream mediator of the α6β4/PI 3-K signaling pathway involved in invasion. The inability of the constitutively active mutant of Rac to significantly increase the invasion of the MDA-MB-435 cells suggests that other PI-3K downstream effectors, in addition to Rac, are important for invasion in these cells.

EXAMPLE X: Involvement of PI 3-K in the α6β4-dependent migration of invasive colon carcinoma cells.

We wished to confirm that the activation of PI 3-K by α6β4 in the MDA-MB-435 transfectants also occurred in a carcinoma cell line that endogenously expresses the α6β4 integrin and that this activation was related to the invasive properties of the cell. For this purpose we chose clone A cells, an invasive colon carcinoma cell line that we have characterized extensively. Clone A cells express relatively high levels of the α6β4 integrin and no α6β1 integrin (Lee et al., *J. Cell Biol.* 117:671-8, 1992). Importantly, these cells use the α6β4 integrin as an adhesion receptor for laminin-1 (Lee et al., *J. Cell Biol.* 117:671-8, 1992; Lotz et al., *Cell Regul.* 1:249-57, 1990), in contrast to the MDA-MB-435/β4 transfectants.

Clone A cells were assayed for their ability to invade Matrigel in the presence of monoclonal antibodies specific for the indicated integrin subunits. Cells were pre-incubated for 30 minutes in the presence of antibodies before addition to the Matrigel-coated wells. After 24 hours at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in previous sections. The data shown are the mean values (+SD) of a

representative experiment done in duplicate. "IgG" indicates non-specific antibody; "β1" indicates mAb 13; "α6" indicates 2B7; "cont" indicates control; and "WT" indicates wortmannin (Figs. 7A and 7B). As shown in Fig. 7A shows that the Matrigel invasion of Clone A colon carcinoma cells is inhibited by α6-specific antibodies.

Recently, we demonstrated that α6β4 integrin performs an essential role in the migration of Clone A cells on laminin-1 by promoting the formation and stabilization of filopodia and lamellae. Based on the data obtained with MDA-MB-435 cells, the prediction can be made that the α6β4-dependent formation of actin-containing motility structures is dependent on PI 3-K and that ligation of α6β4 stimulates PI 3-K activity in Clone A cells. To examine this possibility, Clone A cells were treated with wortmannin (100 nM) prior to their plating on laminin-1 and their behavior was then monitored by video microscopy. Clone A cells were allowed to adhere to laminin-1 in the absence or presence of wortmannin (100 nM) for 45 minutes. Images were obtained at this time using a Nikon Diaphot 300 inverted microscope with phase contrast optics. This microscope was connected to a CCD camera (Dage-MTI), a frame-grabber (Scion) and a 7600 Power Macintosh computer to capture the images. The lamellar area for the cells in each condition was determined using IPlab Spectrum image analysis software. The data shown are the mean values (+SEM) for > 50 cells. Wortmannin had no effect on the attachment of clone A cells to laminin or on their initial spreading. However, wortmannin inhibited the formation of lamellae by 80% (Fig 7B).

Interestingly, the effect of wortmannin on the formation of lamellae was very similar to the effect of a function-blocking α6 antibody that recognizes only the α6β4 integrin in these cells. In the experiment shown in Fig. 7C, Clone A cells were allowed to adhere to laminin-1 in the absence or presence of wortmannin (100 nM) or the α6-specific antibody, 2B7, as

indicated, for 45 minutes. Representative images from cells in each condition are shown. Similarly to wortmannin, the α6 antibody inhibited the formation of lamellae.

To examine whether α6β4 activates PI 3-K in clone A cells, we performed *in vitro* kinase assays on cell extracts prepared from clone A cells that had attached to laminin-1 in the presence of either non-specific IgG or an α6-specific antibody for 45 minutes. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine mAb 4G10 and Protein A sepharose for 3 hours. After washing, the beads were resuspended in kinase buffer and incubated for 10 minutes at room temperature. The labeled lipids were resolved by thin layer chromatography. The D3-phosphoinositides are indicated by arrows in Fig. 8A.

Fig. 8B shows a densitometric analysis of the amount of radiolabeled PtdIns-3,4,5-P3 for each experimental condition. The adhesion-dependent levels of PtdIns-3,4,5-P3 were compared to the level observed for the cells that were maintained in suspension which was given the value of 1. The value from this ratio was determined to be the relative PI 3-K activity stimulated by adhesion. The data shown are the mean values (+SEM) from 2 experiments.

As shown in Figs. 8A and 8B, PI 3-K activity was increased after attachment to laminin-1 but this increase was inhibited when the α6β4 receptor was blocked by the α6 antibody. Although clone A cells still adhere to laminin-1 in the presence of the α6 antibody using the α2β1 integrin (Fig. 7C), there was no increase in PI 3-K activity in these cells compared to cells maintained in suspension. Therefore, a differential ability of the α6β4 and β1 integrins to activate PI 3-K is evident in clone A cells, as was observed in the MDA-MB-435/β4 transfectants.

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In summary, α6β4 is required for PI 3-K activation and formation of lamellae in response to laminin-1 attachment in clone A colon carcinoma cells; functions that are required for the invasion of these cells.

EXAMPLE XI: α6β4-dependent invasion requires protein kinase C activity.

To determine whether members of the protein kinase C (PKC) serine/threonine kinase family are necessary for carcinoma invasiveness, we tested whether Calphostin C, a specific inhibitor of the PKC family, could inhibit carcinoma cell motility in the Matrigel invasion assay. As shown in Figure 9A, Calphostin C inhibits the invasion of both the mock (6D2) and β4 (5B3) MDA-MB-435 breast carcinoma transfectants.

Members of the PKC family are differentially regulated by diacylglycerol and the tumor promoter phorbol myristal acetate (PMA): the conventional isoforms are down-regulated by long term PMA exposure, whereas the novel and atypical PKC isoforms are not down-regulated by PMA. As shown in Figure 9B, overnight incubation with PMA almost completely inhibited the ability of the mock transfectants to invade ("PMA+" indicates that in addition to the overnight incubation, fresh PMA was added to the Matrigel as well). In contrast, the MDA-MB-435/β4 transfectants (5B3) were only partially inhibited by PMA. Furthermore, the invasiveness of these PMA-resistant cells remains sensitive to Calphostin C inhibition, indicating that a PMA-insensitive PKC isoform is required for invasion (Figure 9C). We conclude that α6β4 activates a non-conventional PKC isoform that is important for invasion. Significantly, the atypical PKC isoforms are known to be regulated by the lipid products of PI 3-K. Hence, we further conclude that atypical PKC isoforms are likely to be important downstream effectors of the PI 3-K signaling pathway that promotes carcinoma invasion, although it is

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likely that novel and conventional PKC isoforms also are involved. Such PKC isoforms provide important biological targets by which to modulate tumor invasiveness.

EXAMPLE XII: α6β4 integrin-dependent motility involves increased cAMP phosphodiesterase activity.

We noted that β4 integrin-transfected MDA-MB-435 cells exhibited higher cAMP phosphodiesterase activity and lower cAMP levels than non-transfected cells. Significantly, β4 integrin-enhanced motility was blocked by the phosphodiesterase inhibitors IBMX and rolipram, and by forskolin.

Fig. 10 shows the cAMP phosphodiesterase activity of MDA-MB-435 clones. MDA-MB-435 cells transfected with either full length β4 integrin subunit (3A7, 5B3) or vector alone (6D7) in suspension or plated on laminin-1 or collagen I were treated for 15 min. with 50 μM Forskolin or 100 nM LPA as noted. Cells were harvested and the cytosolic fraction was assayed for PDE activity as described in Example I. Bars represent standard error of at least 4 separate determinations. Phosphodiesterase activity is highest in β4 integrin-expressing clones.

Fig. 11 shows that the phosphodiesterase inhibitor, IBMX, decreases LPA-stimulated chemotaxis of MDA-MB-435 cells. Cells suspensions of MDA-MB-435 subclone 5B3 (β4 transfected; squares) or 6D7 (mock transfected; circles) were treated with the indicated concentration of IBMX for 30 min. prior to use in LPA chemotaxis assay as described in Example I. Bars represent standard deviation of triplicate determinations.

EXAMPLE XIII: α6β4 integrin functions in carcinoma cell migration by mediating the formation and stabilization of actin-containing mobility structures.

Functional studies of the α6β4 integrin have focused primarily on its role in the organization of hemidesmosomes, stable adhesive structures that anchor cells in place by physically interacting with the intermediate filament cytoskeleton. However, the association of α6β4 integrin expression with carcinoma invasion is inconsistent with its established role in the formation of hemidesmosomes, and in fact, the notion that α6β4 integrin is necessary for both hemidesmosome formation and cell motility is counterintuitive.

Clone A colon carcinoma cells, which express α6β4 integrin, but not α6β1 integrin, exhibit dynamic adhesion and motility on laminin-1. Their migration is characterized by filopodial extension and stabilization, followed by lamellae that extend in the direction of stabilized filopodia. A functional blocking antibody for α6β4 integrin inhibited clone A migration on laminin-1, as well as inhibiting filopodial formation and stabilization and lamella formation, leading to the unexpected conclusion that α6β4 integrin must be a component of the clone A cell motility apparatus.

The unexpected finding that α6β4 integrin appeared necessary for clone A motility led us to ask whether α6β4 integrin could interact with the actin cytoskeleton, a dynamic structure that is essential for cell migration. Such an interaction would be surprising, as α6β4 integrin is thought to associate only with the more stable intermediate filaments, such as cytokeratins. Contrary to our expectations, a striking co-localization of α6β4 integrin and F-actin was observed in filopodia and at the distal sites of lamellae.

Figure 12 shows that α6β4 integrin co-localizes with F-actin in filopodia of clone A cells on laminin-1. Cells plated on either laminin-1 (Fig. 12 A-D and F) or collagen I (Fig. 12E) at 35°C for 1 h. were processed for

double immunofluorescence using the rat GoH3 mAb followed by a rhodamine-conjugated anti-rat antibody and FITC-conjugated phalloidin. The confocal images shown represent optical sections of the ventral surface of the cells (Fig. 12A and 12C) GoH3; (Figs. 12B-12F, Phalloidin). Fig. 12A and 12B demonstrate co-localization of α6β4 and F-actin in a group of filopodia. Fig. 12D shows the formation of actin cables on the top lamella that project into filopodia. These filopodia are enriched in α6β4 (Fig. 12C). Fig. 12E shows the presence of polygonal actin cables in clone A cells plated on collagen I. In Fig. 12F, the cells were incubated with 2B7 antibody for 30 min. before fixation. Note the disappearance of actin cables (remaining protrusions are presumably retraction fibers). The bar shown at the bottom of Fig. 13F represents 10 μm.

Not only did we observe α6β4 integrin to be co-localized with F-actin in filopodia and lamellae, even more surprisingly, we found that α6β4 integrin did not co-localize with cytokeratins in filopodia and distal sites of lamellae. Instead, cytokeratin staining was concentrated largely in the cell body and in proximal portions of lamellae.

To study the interaction of the α6β4 integrin with the cytoskeleton in more detail, we used an *in situ* extraction scheme that solubilizes a protein in a manner that correlates with the cytoskeletal associations of the protein. Clone A cells adherent to laminin-1 were extracted with either a 0.5% Triton X-100 buffer that removes most of the soluble protein and phospholipid but not the actin and intermediate filament cytoskeletons, or a two-detergent buffer (1.0% Tween-40/0.5% deoxycholate) that removes the bulk of the actin cytoskeleton but not intermediate filaments and associated proteins. Subsequent to extraction, the cells were fixed and costained with integrin-specific mAbs (GoH3 or K20) and cytoskeletal-specific reagents (phalloidin or pan-cytokeratin mAbs). Extraction of clone A cells with the Triton X-100 buffer

revealed that the α6β4 and F-actin co-localization observed in unextracted cells is preserved in clusters at proximal sites in filopodia, as well as at the roots of filopodia that project into the lamellae (Fig. 13A and B). Several of these co-localization sites were also the origins of actin filament bundles (Fig. 13A and B). In contrast, α6β4 did not co-localize with cytokeratins in filopodia and distal sites of many of the lamellae either in unextracted cells or after the Triton X-100 buffer extraction. These results suggest that α6β4 is retained at the cell edges because of its association with actin and not with cytokeratins. In agreement with this possibility, these marginal areas of actin-associated α6β4 integrin were removed by the Tween/deoxycholate buffer.

The data presented above strongly support an association of the α6β4 integrin with actin filaments in clone A cells. To obtain biochemical evidence for this association, we used the actin-severing protein gelsolin to assess whether severing actin filaments would liberate α6β4. Clone A cells adherent to laminin-1 were extracted with the 0.5% Triton X-100 buffer to remove soluble proteins and then treated with gelsolin. The proteins liberated from extracted cells treated with gelsolin, as well as from extracted cells treated with the buffer alone, were analyzed for the presence of α6β4 by immunoprecipitation with the α6-specific antibody 2B7 and subsequent immunoblotting with a polyclonal antibody specific for the β4 cytoplasmic domain. In the experiment shown in Fig. 14, cells were plated on laminin-1 and incubated for 1 h. at 37°C. After permeablization with a Triton X-100 buffer, the cells were incubated with either gelsolin (g) or control buffer (C) for 30 min. The gelsolin-liberated fraction was immunoprecipitated with an α6-specific antibody (2B7), subjected to SDS-PAGE, and immunoblotted with a β4-specific polyclonal antibody. An aliquot of the gelsolin-liberated fraction was subjected to SDS-PAGE and stained with Coomassic blue to detect the 43-kD actin band that was evident in the gelsolin-treated but not the control cells.

Fig. 14 shows that the 200-kD β4 subunit was liberated from gelsolin-treated cells but not from cells treated with buffer alone. Moreover, the material obtained from the gelsolin-treated cells but not the control cells was enriched in actin.

We explored the possibility that the functional and topographical properties of the α6β4 integrin observed in clone A cells could be extended to other carcinoma cells that express α6β4. The CCL-228 and MIP-101 colon carcinoma cells have been shown previously to express the α6 integrin subunit exclusively associated with β4, while the MDA-231 breast carcinoma cells express primarily the α6β4 heterodimer. These carcinoma cells were analyzed by indirect immunofluorescence using an anti-β4 antibody (A9) and FITC-phalloidin.

In the experiment shown in Fig. 15, MIP-101 (Fig. 15A and 15B), MDA-MB-231 (Fig. 15C and 15D), and CCL-228 (Fig. 15E) carcinoma cells were analyzed by double immunostaining with the β4-specific A9 antibody (Fig. 15A, 15C and 15D) and FITC-phalloidin (Fig. 15B and 15C). Note the concentration of α6β4 in the actin nodes present in the filopodia of MIP-101 cells (Fig. 15A and 15B, arrowheads) and the distribution of α6β4 in filopodia, retraction fibers, and lamellae of MDA-231 and CCL-228 cells. (Fig. 15F) an α6-specific antibody inhibits formation of lamellae in CCL-228 cells. Cells were plated on laminin-1 in the presence or absence of 2B7 for 1 h. The cells were photographed, and their lamellar area (μm^2/cell) was determined by digital image analysis. Fifty cells were analyzed for each condition. The bar in panel 15A represents 20 μm. The error bar in Fig. 15F represents SEM.

Although these carcinoma cells differed markedly in their morphology on laminin-1, all of them exhibited a fine grainy pattern of β4 staining on their ventral surfaces (Fig. 15A-15E). More specifically, β4 and actin was seen in these structures similar to the results obtained with clone A

cells. Interestingly, MIP-101 cells exhibited long filopodia with distinct "actin nodes" that were enriched in β4 staining. (Fig. 15A and 15B).

We also explored the function of the α6β4 integrin in the dynamic behavior of CCL-228 cells on laminin-1 using the function-blocking 2B7 antibody. As shown in Fig. 15F, 2B7 marketedly inhibited the formation of lamellae, but it did not affect cell attachment. Similar results were obtained with MIP101 and MDA-MB-231 cells. These data indicate that the interaction of α6β4 with actin-containing motility structures is a frequent phenomenon in carcinoma cells.

EXAMPLE XIV: Wound-induced motility of T84 colon carcinoma cells is dependent upon PI 3-K.

Disruptions in the mucosal lining of the gastrointestinal tract reseal by a process termed restitution. This mode of wound healing results in the repair of superficial wounding in the mucosa by the process cell migration. More specifically, epithelial cells from the edges of wounds are thought to migrate as a sheet into the wound. When the cells are within the wound area, they rapidly reform cell contacts and re-establish barrier functions. Although the mechanism of intestinal epithelial restitution is poorly understood, it likely involves the altered regulation and expression of molecules involved in cell migration. Such molecules include cytoskeletal proteins, adhesion receptors, and extracellular matrix glycoproteins. Human T84 colon carcinoma cells provide a good model for the study of the biological processes, e.g., cell migration, that occur during wound healing.

Fig. 16A-16F are photomicrographs showing that lamellae extension during T84 cell wound healing is wortmannin-sensitive. Fig. 16A is at time zero. A wound is made in a confluent monolayer of T84 cells, a well-differentiated, polarized human colon carcinoma cell line. The wound was

made by aspiration using a narrow gauge Pasteur pipet tip, and is 7577 μm^2, equaling approximately 150 cells. These wounds heal by a process similar to that observed when rabbit stomach mucosa is damaged. Cells around the wound flatten and then extend lamellae, large thin protrusions involved in cell migration. Fig. 16B is at 50 minutes post-wounding. At this time, 100 nm of wortmannin, an inhibitor of PI3 kinase, is added. The wound is 2802 μm^2 in diameter. The lamellae, marked by an L, extend into the denuded area. The wound has sealed to 63% of its original diameter. Fig. 16C is at 54 minutes post-wounding. Four minutes after wortmannin is added, lamellae begin to collapse. Fig. 16D is at 62 minutes post-wounding. Lamellae continue to collapse, and the wound contains a prominent, thickened edge of a retracting lamellae (lower edge, approximately 6:00). Fig. 16E is at 68 minutes post-wounding. Eighteen minutes after the addition of wortmannin, lamellae along the wound edge begin to extend once again. The wound diameter is now 2201 μm^2. During the last 18 minutes, while lamellae were collapsing, the wound sealed 22%. Fig. 16F is at 86 minutes post-wounding. Lamellae extend into the wound from most cells adjacent to the edge. The wound is now 1044 μm^2 in diameter. During the last 18 minutes, while lamellae were extending, the wound sealed about 50%. In summary, these data show that lamellae extension is required to maintain the rate of wound closure. Furthermore, the formation of lamellae requires signaling by PI 3-K.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is:

RNSDOCID: <WO 9935283A1 I >

Claims

1. A method of identifying a compound that modulates the motility or invasiveness of a cell, said method comprising the steps of:

a) exposing a sample to a test compound, wherein said sample comprises PI 3-K, PKC, cAMP-PDE, or α6β4 integrin, and

b) assaying for altered biological activity of PI 3-K, PKC, cAMP-PDE, or α6β4 integrin, wherein a decrease in said biological activity, relative to the biological activity of a sample not exposed to said compound, indicates a compound that decreases cell motility or cell invasiveness, and wherein an increase in said biological activity, relative to the biological activity of a sample not exposed to said compound, indicates a compound that increases cell motility or cell invasiveness.

2. The method of claim 1, wherein said PI 3-K, PKC, or cAMP-PDE is constitutively activated.

3. The method of claim 1, wherein said PI 3-K, PKC, cAMP-PDE, or α6β4 integrin is within a cell.

4. The method of claim 1, wherein said sample comprises cell lysate or cell extract.

5. The method of claim 1, wherein said PKC is an atypical PKC.

6. The method of claim 1, said method further comprising a step wherein said level of α6β4 integrin or said biological activity of PI 3-K, PKC,

cAMP-PDE, or α6β4 integrin is increased or decreased prior to exposing said sample to said test compound.

7. A method of identifying a compound that modulates the motility or invasiveness of a cell, said method comprising the steps of:

a) modulating the biological activity of PI 3-K, PKC, cAMP-PDE, Akt, or α6β4 integrin in a cell,

b) exposing said cell to a test compound, and

c) assaying for altered motility or invasiveness of said cell exposed to said compound, wherein a decrease in said motility or said invasiveness, relative to the motility or invasiveness of a cell not exposed to said compound, indicates a compound that decreases cell motility or cell invasiveness, and an increase in said motility or said invasiveness, relative to a cell not exposed to said compound, indicates a compound that increases cell motility or cell invasiveness.

8. The method of claim 7, wherein said biological activity is increased by introducing constitutively active PI 3-K, PKC, or cAMP-PDE into said cell.

9. The method of claim 7, wherein said biological activity is decreased by introducing dominant-negative PI 3-K, PKC, or cAMP-PDE into said cell.

10. The method of claim 7, wherein said PKC is an atypical PKC.

11. A method of decreasing the invasiveness of a cell or decreasing the predisposition to developing an invasive cell, said method comprising

identifying the presence of at least one invasive cell or at least one cell with a predisposition to developing invasiveness, and exposing at least one invasive cell or at least one cell with a predisposition to developing invasiveness to a compound that decreases the biological activity of PI 3-K, PKC, cAMP-PDE, or α6β4 integrin, or increases the biological activity of Akt.

12. The method of claim 11, wherein said PKC is an atypical PKC.

13. The method of claim 11, wherein said compound is an antibody that specifically binds PI 3-K, PKC, cAMP-PDE, or α6β4 integrin.

14. The method of claim 11, wherein said compound is antisense nucleic acid that specifically hybridizes with nucleic acid encoding PI 3-K, PKC, or cAMP-PDE.

15. The method of claim 11, wherein said compound is dominant-negative PI3-K, dominant-negative PKC, dominant-negative cAMP-PDE, or constitutively-activated Akt.

16. The method of claim 11, wherein said compound is wortmannin.

17. The method of claim 11, wherein said compound is a substrate for PI 3-K, PKC, or cAMP-PDE.

18. The method of claim 11, wherein said cell is a neoplastic cell.

19. The method of claim 18, wherein said neoplastic cell is a colon carcinoma cell, a breast carcinoma cell, a prostate carcinoma cell, a cervical carcinoma cell, a uterine carcinoma cell, a testicular carcinoma cell, a liver carcinoma cell, an ovarian carcinoma cell, a renal carcinoma cell, a bladder carcinoma cell, a lung carcinoma cell, a laryngeal carcinoma cell, a squamous carcinoma cell, or a salivary gland carcinoma cell.

20. A method of increasing the motility of a cell, said method comprising exposing said cell to a compound that increases the biological activity of of PI 3-K, PKC, cAMP-PDE, or $\alpha 6\beta 4$ integrin.

21. The method of claim 20, wherein increasing said motility of said cell results in increased wound healing.

22. The method of claim 20, wherein said PKC is an atypical PKC.

23. The method of claim 20, wherein said cell is an epithelial cell.

24. The method of claim 20, wherein said epithelial cell is an epidermal epithelial cell, an oral epithelial cell, a nasal epithelial cell, a gastrointestinal epithelial cell, a rectal epithelial cell, or an anal epithelial cell.

25. The method of claim 21, wherein said wound results from a gastric ulcer, a duodenal ulcer, inflammatory bowel disease, ulcerative colitis, Crohn's disease, hemorrhoids, surgery, cancer, irradiation, exposure to toxic compounds, or physical trauma.

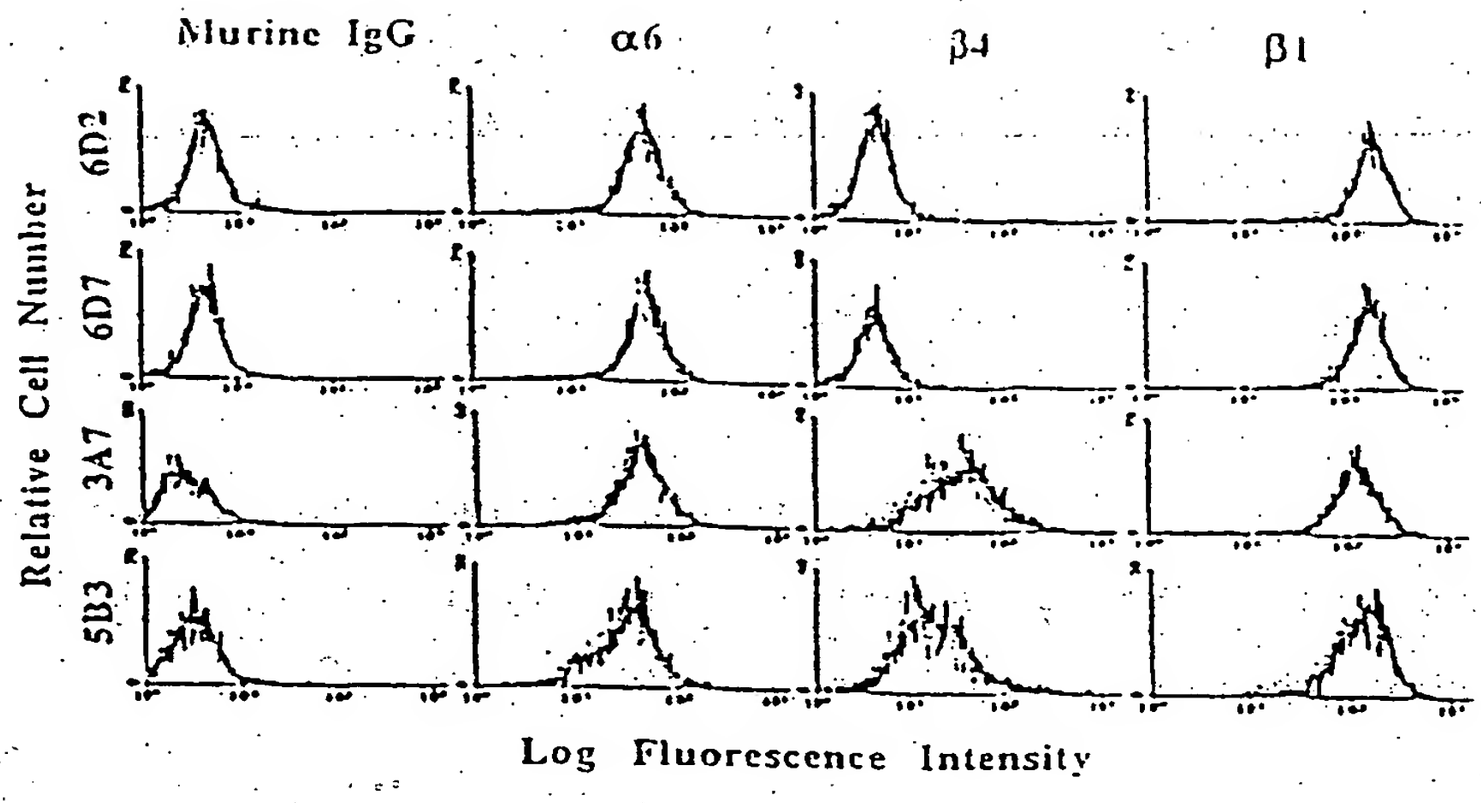
Murine IgG
α6
β4
β1
6D2
6D7
3A7
5B3
Relative Cell Number
Log Fluorescence Intensity

FIG. 1

FIG. 2A

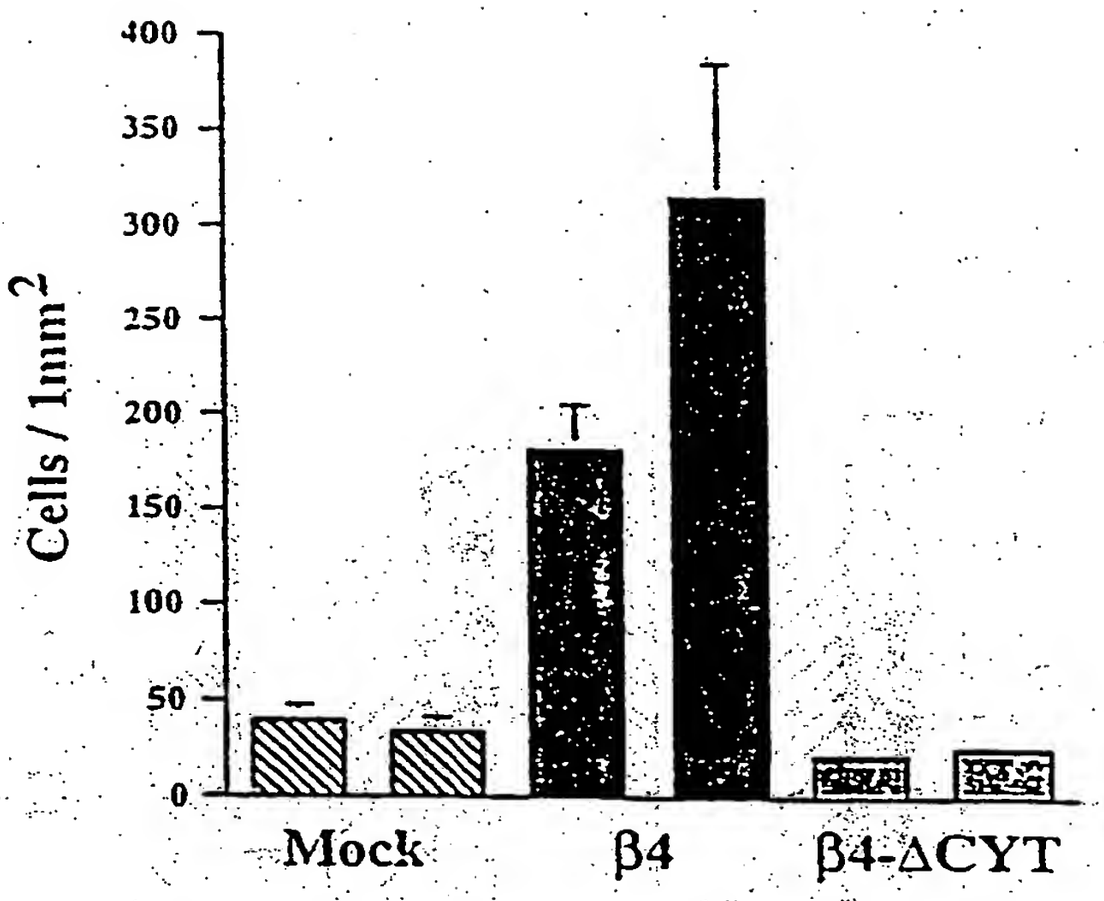
400
350
300
250
200
150
100
50
0
Cells / 1mm2
Mock
β4
β4-ΔCYT

FIG. 2B

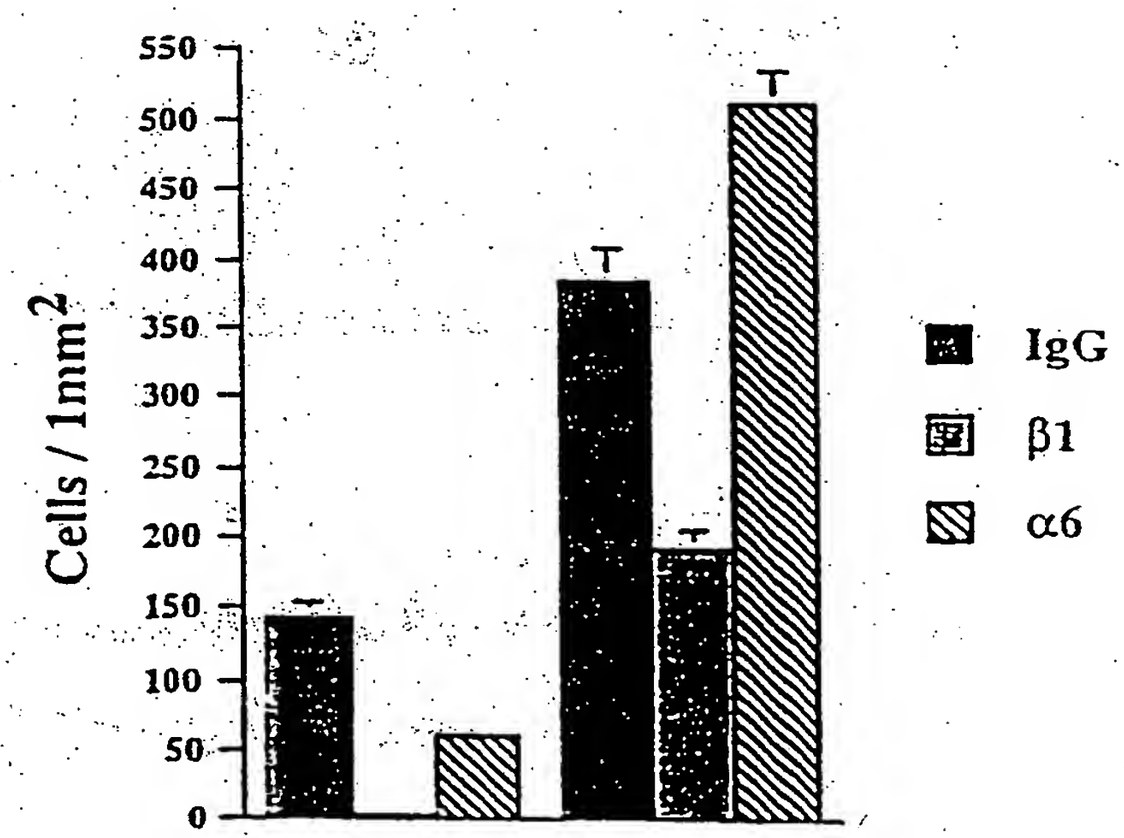
550
500
450
400
350
300
250
200
150
100
50
0
Cells / 1mm2
IgG
β1
α6
Mock
β4

FIG. 3A

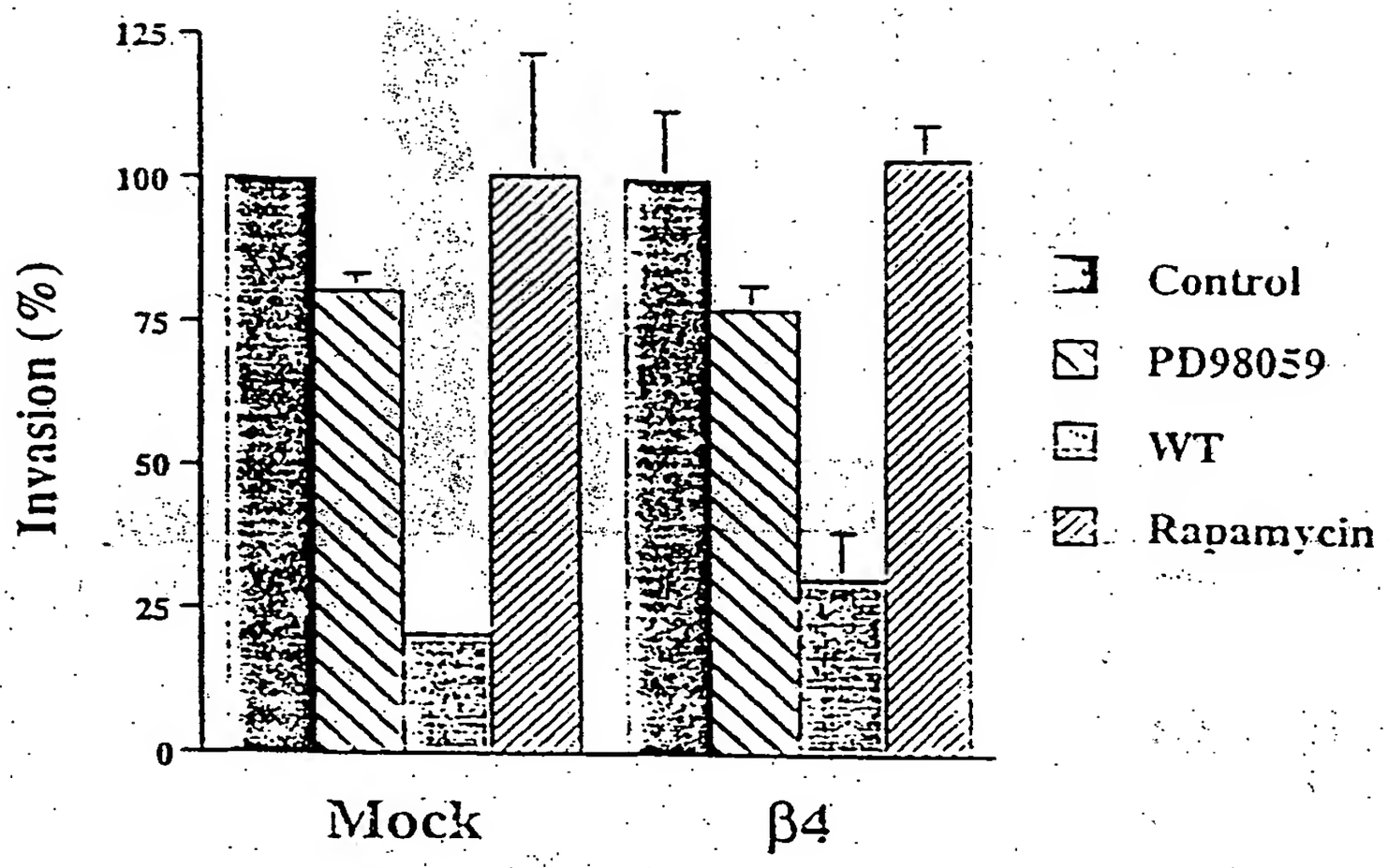
125
100
75
50
25
0
Invasion (%)
Mock
β4
Control
PD98059
WT
Rapamycin

FIG. 3B

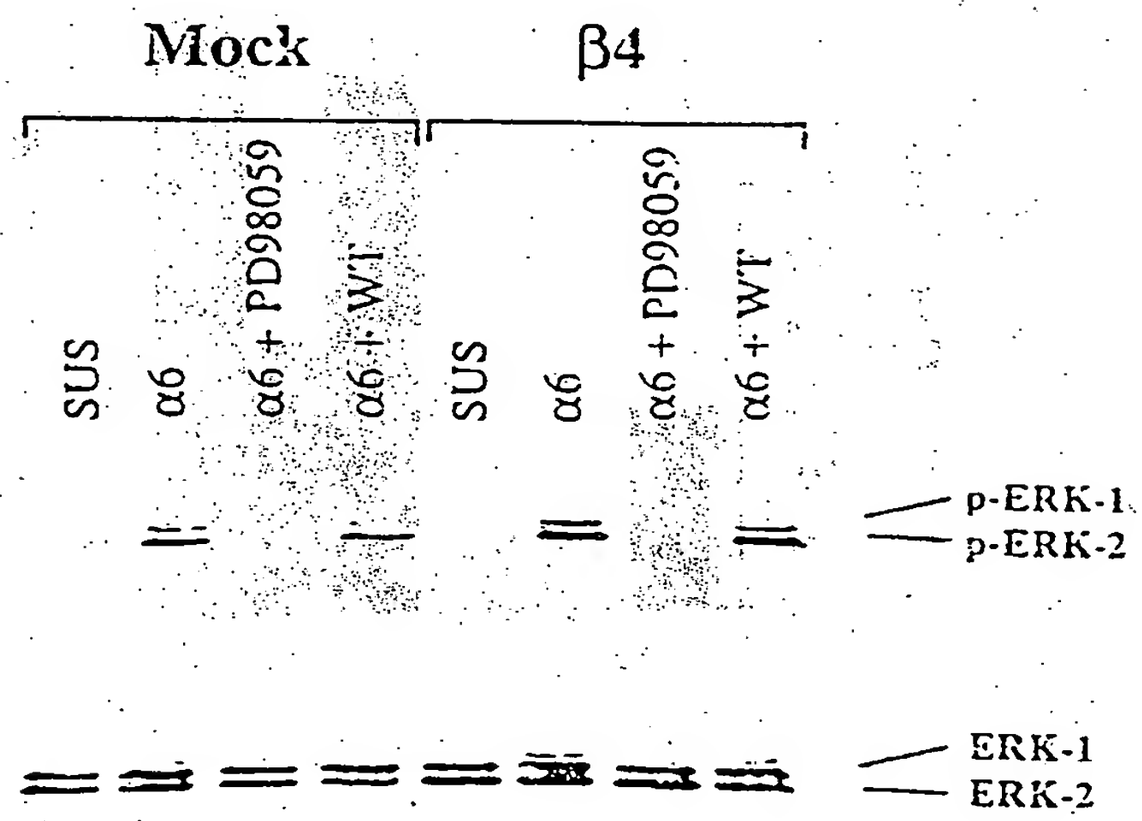
Mock
β4
SUS
α6
α6 + PD98059
α6 + WT
SUS
α6
α6 + PD98059
α6 + WT
p-ERK-1
p-ERK-2
ERK-1
ERK-2

FIG. 4A

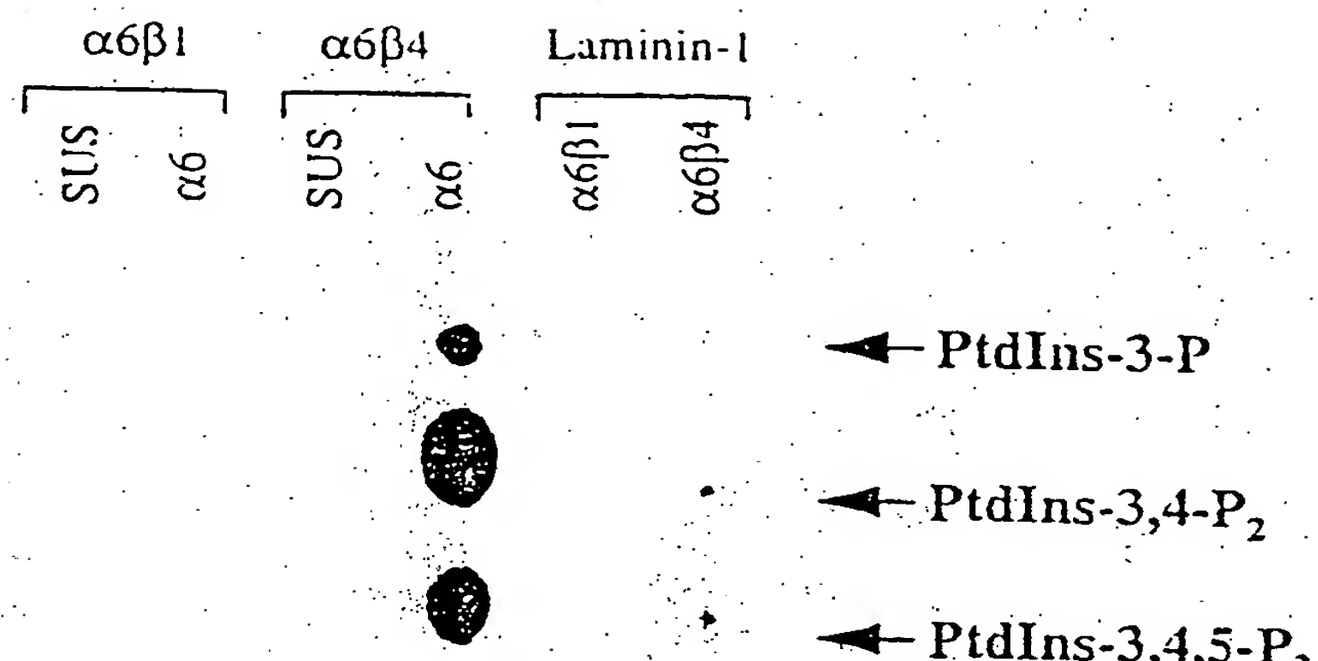

FIG. 4B

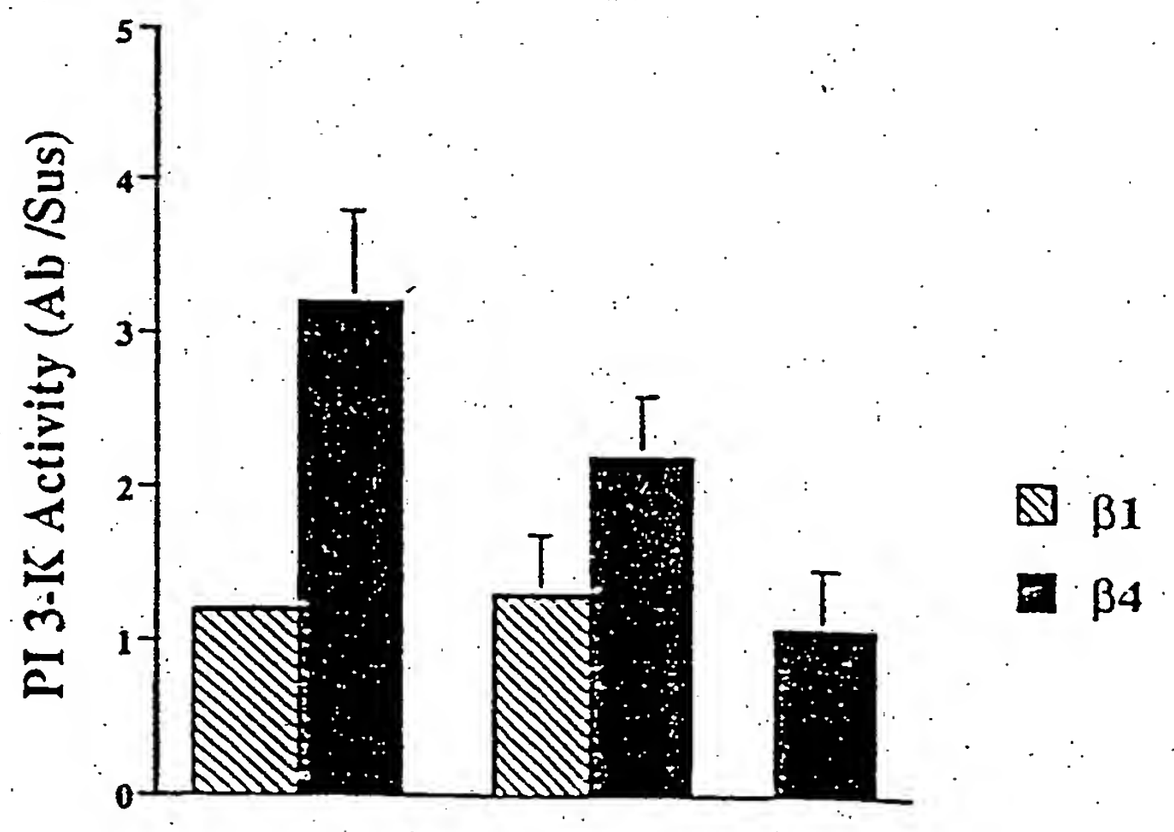

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FIG. 5A

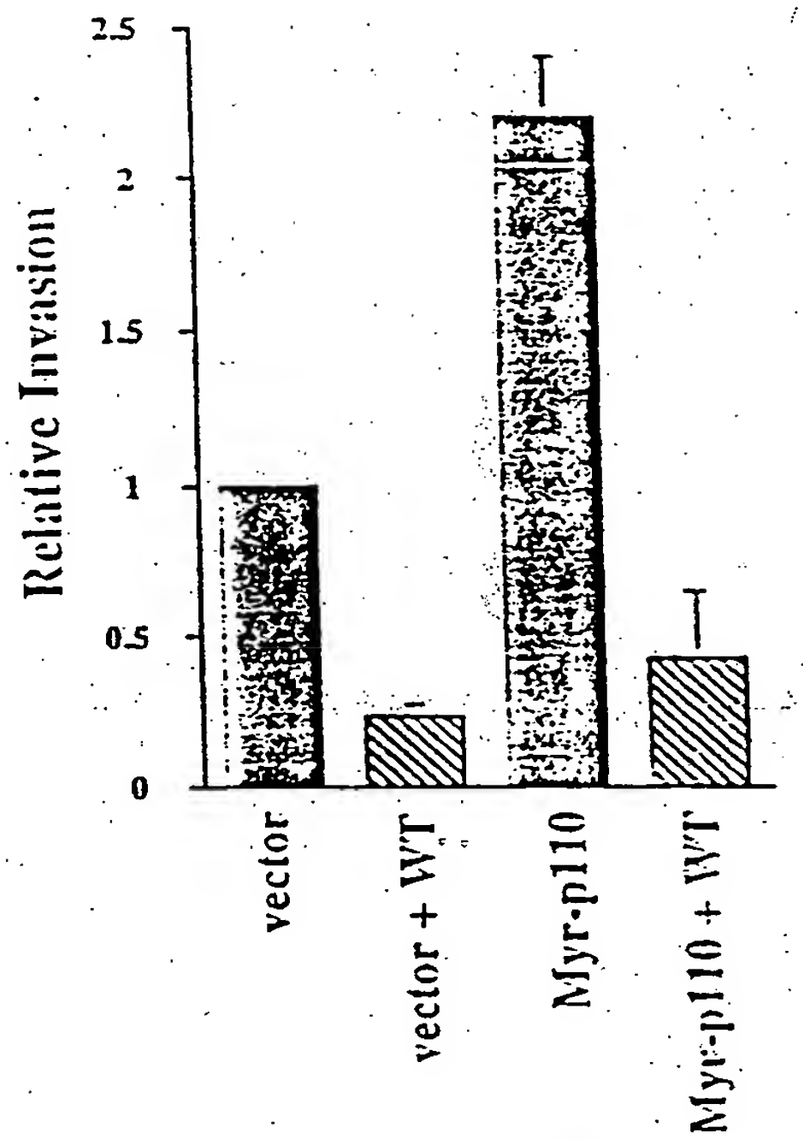
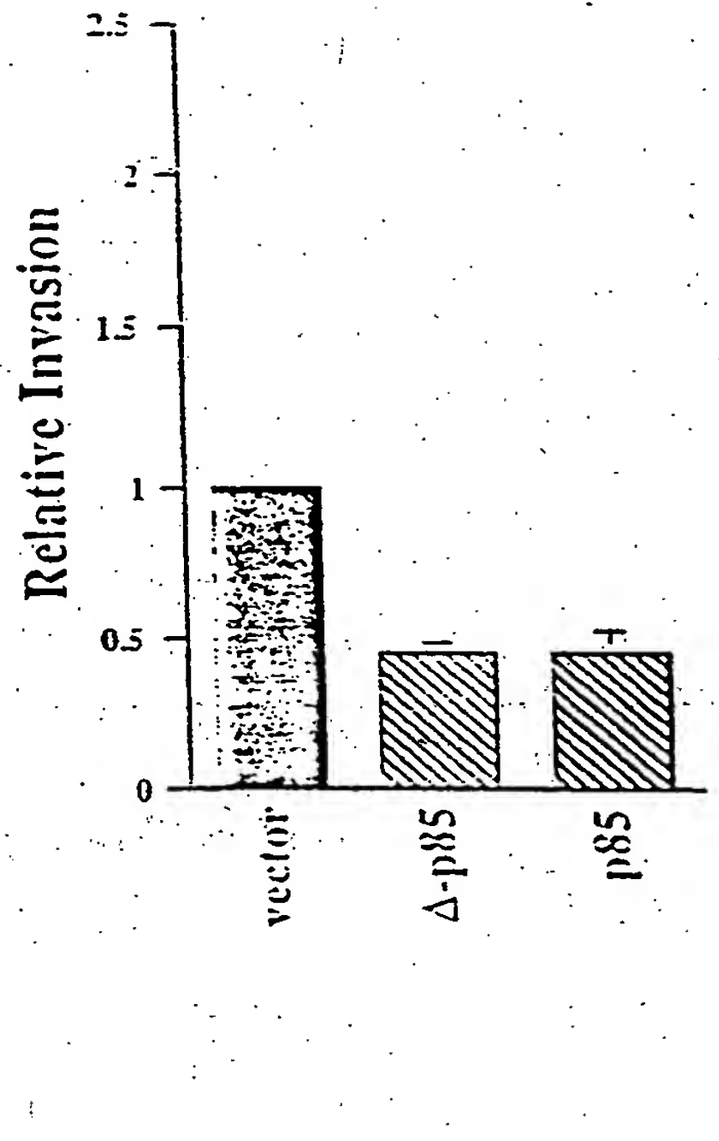

Relative Invasion
2.5
2
1.5
1
0.5
0
vector
vector + WT
Myr-p110
Myr-p110 + WT

FIG. 5B


Relative Invasion
2.5
2
1.5
1
0.5
0
vector
Δ-p85
p85

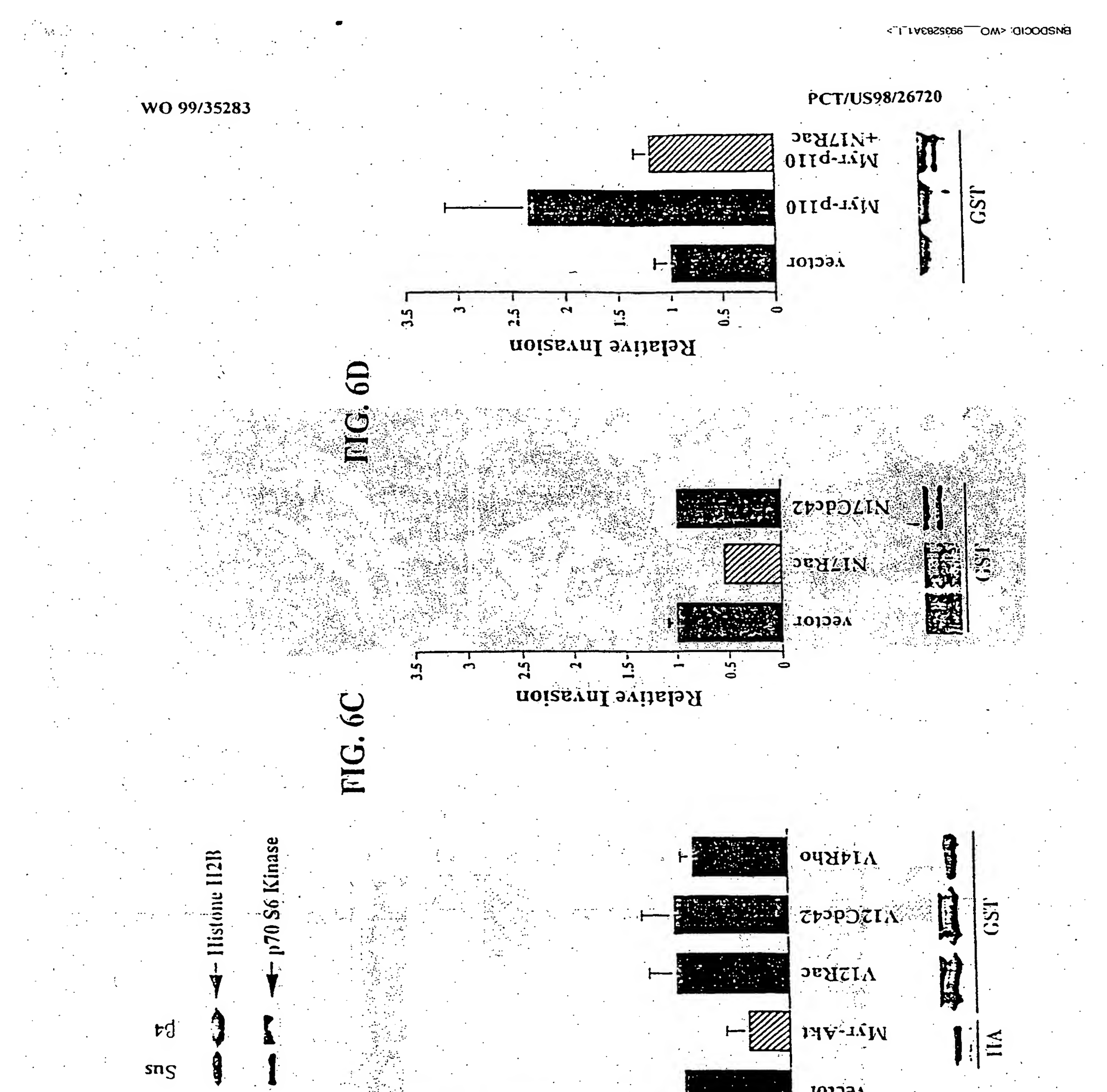


FIG. 6D

FIG. 6C

FIG. 6B

FIG. 6A

FIG. 7A

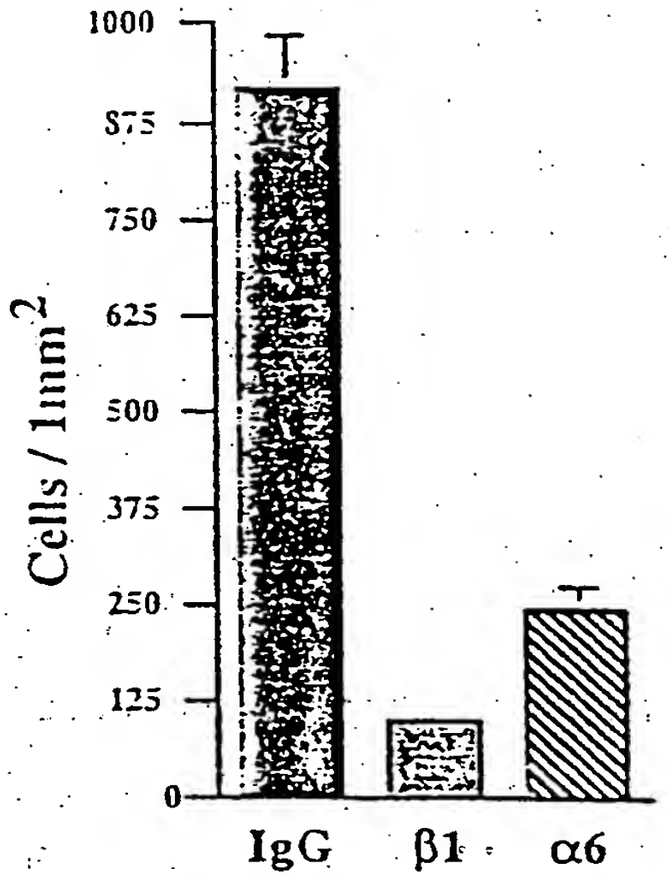


FIG. 7B

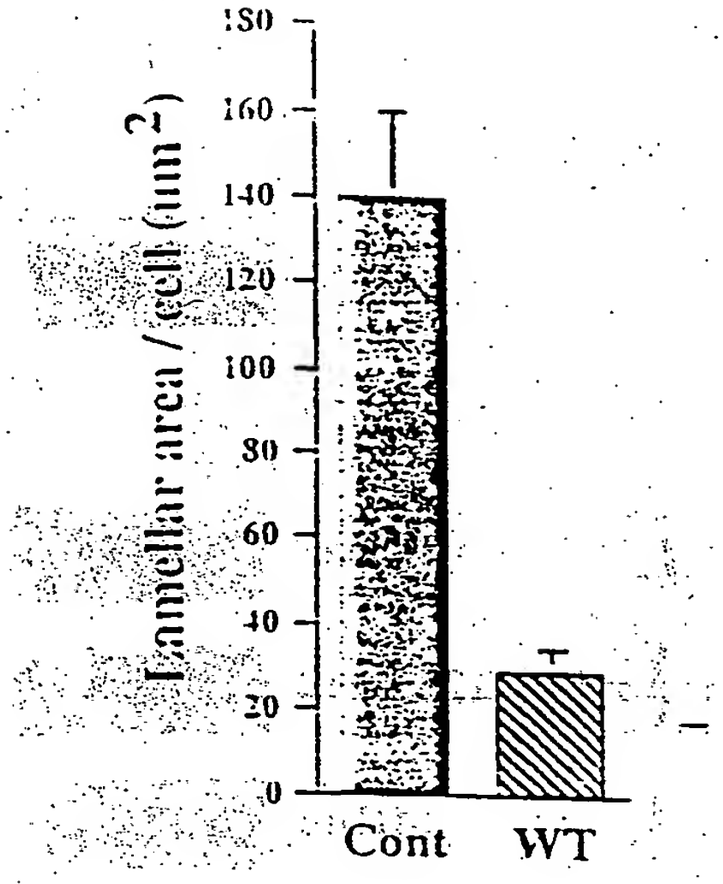


FIG. 7C

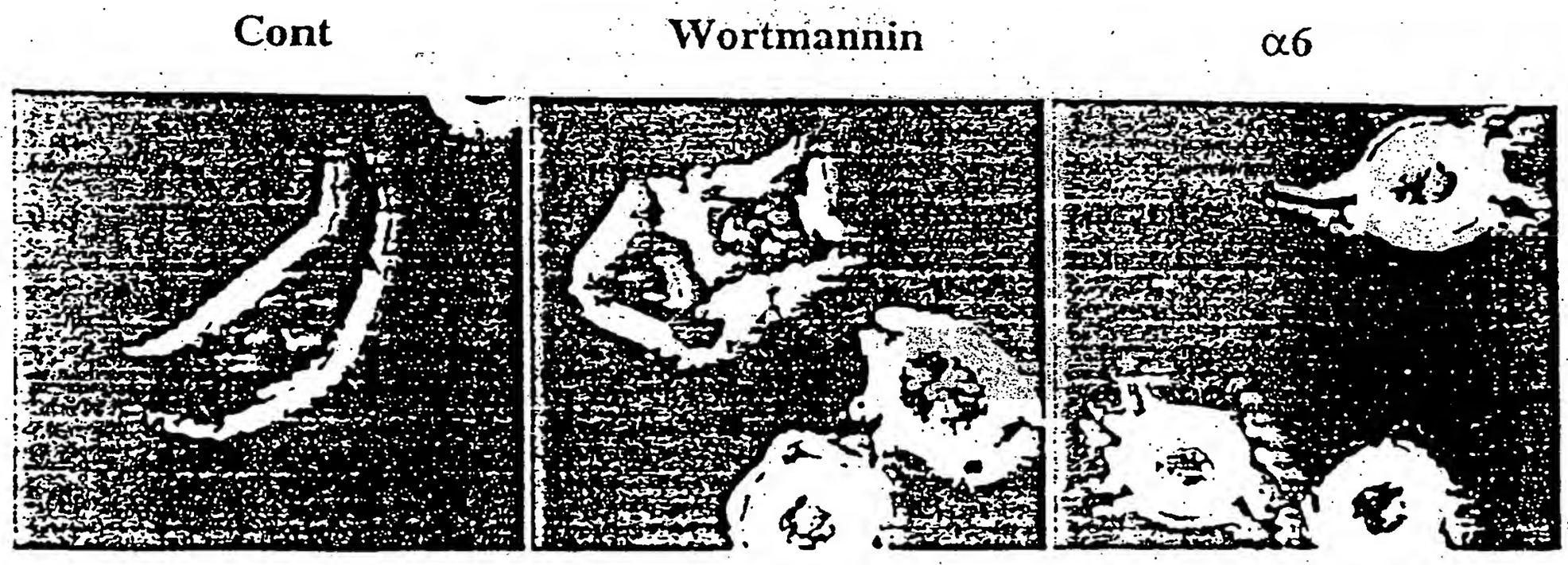


FIG. 8A

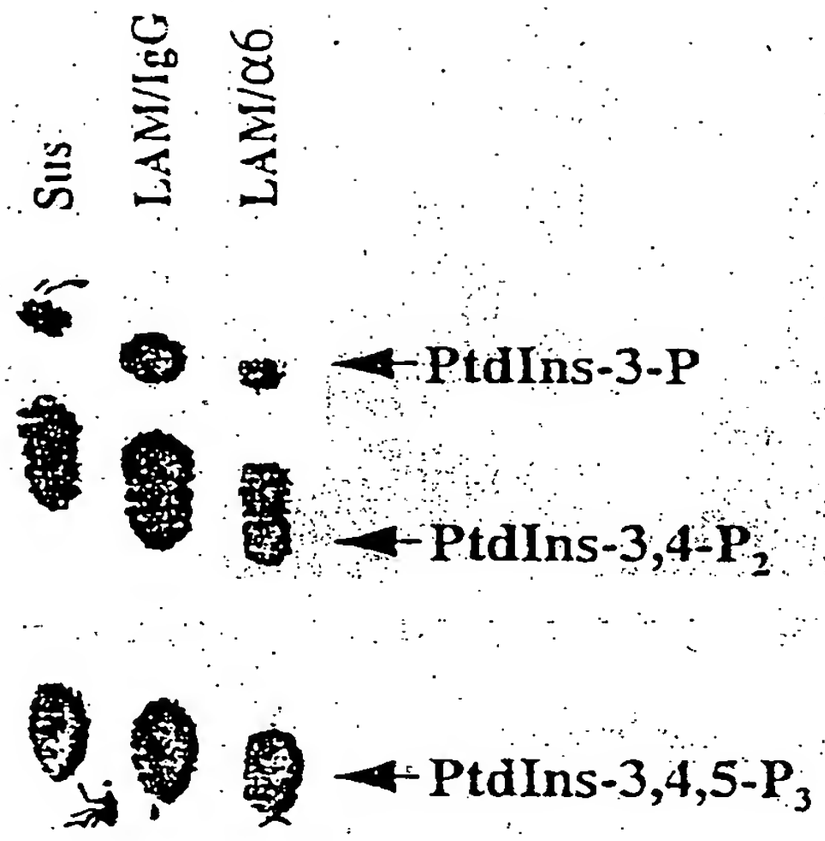
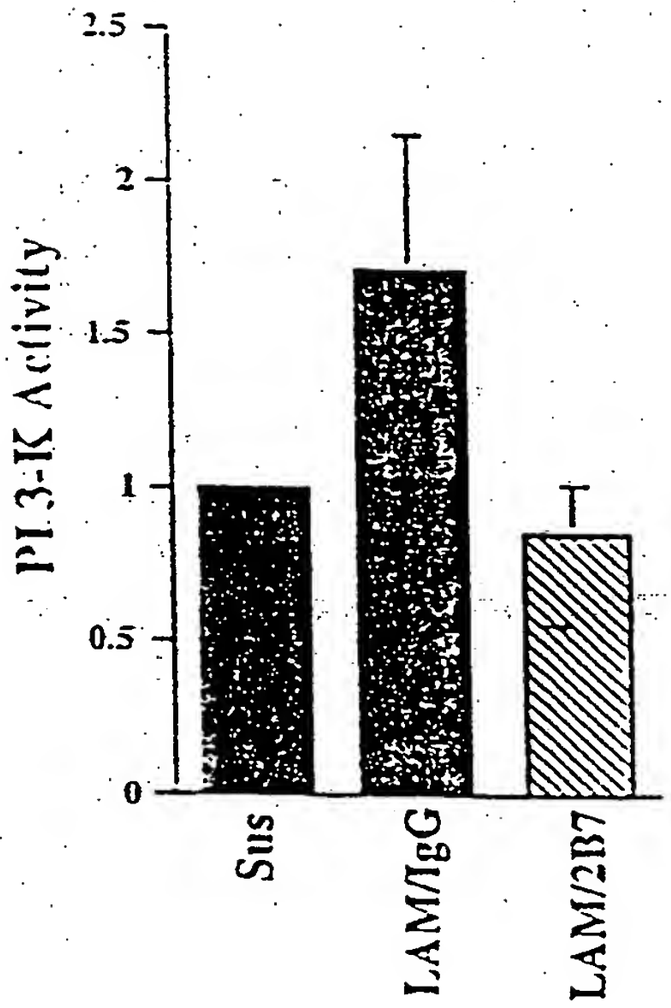


FIG. 8B



BNSDOCID: <WO___9935283A1_I_>

FIG. 9A

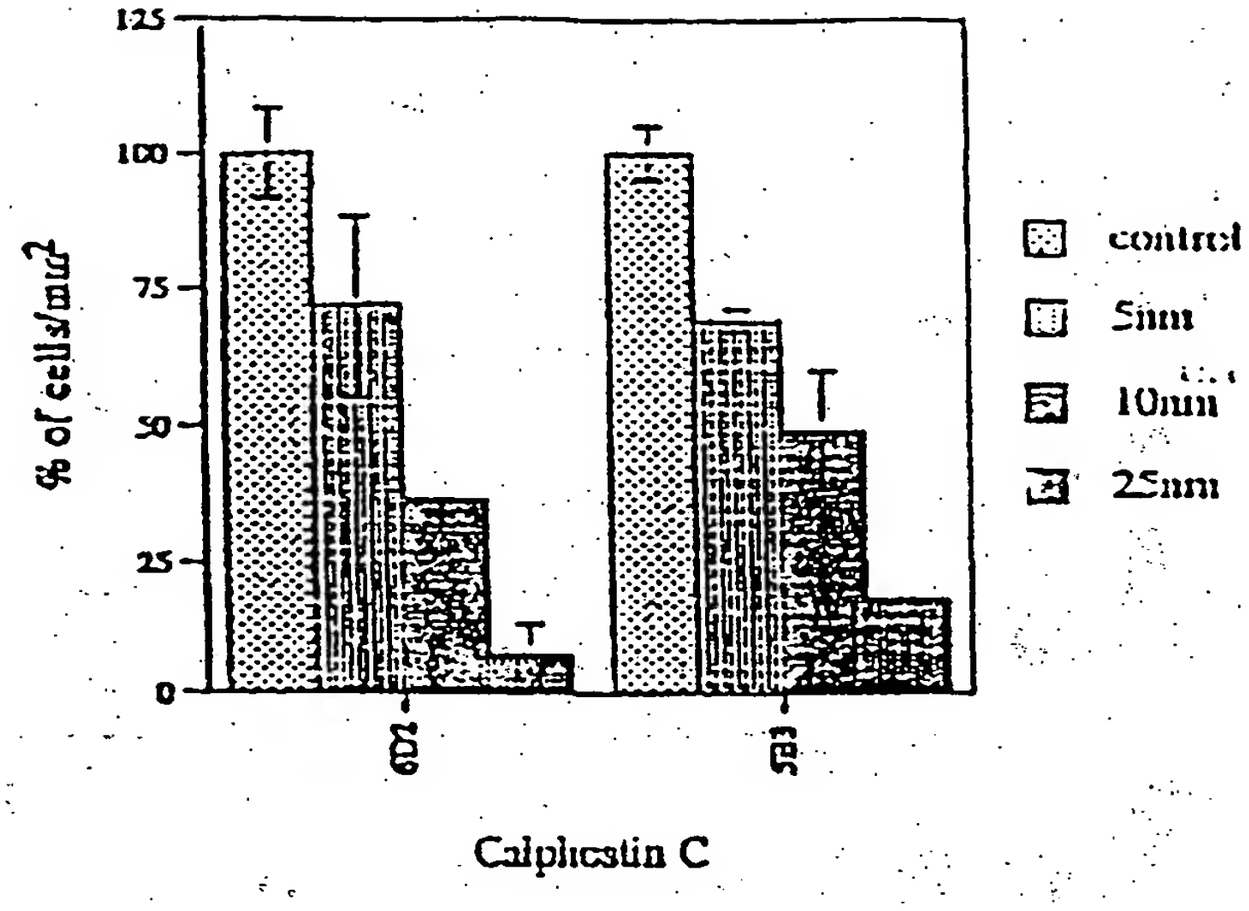


FIG. 9B

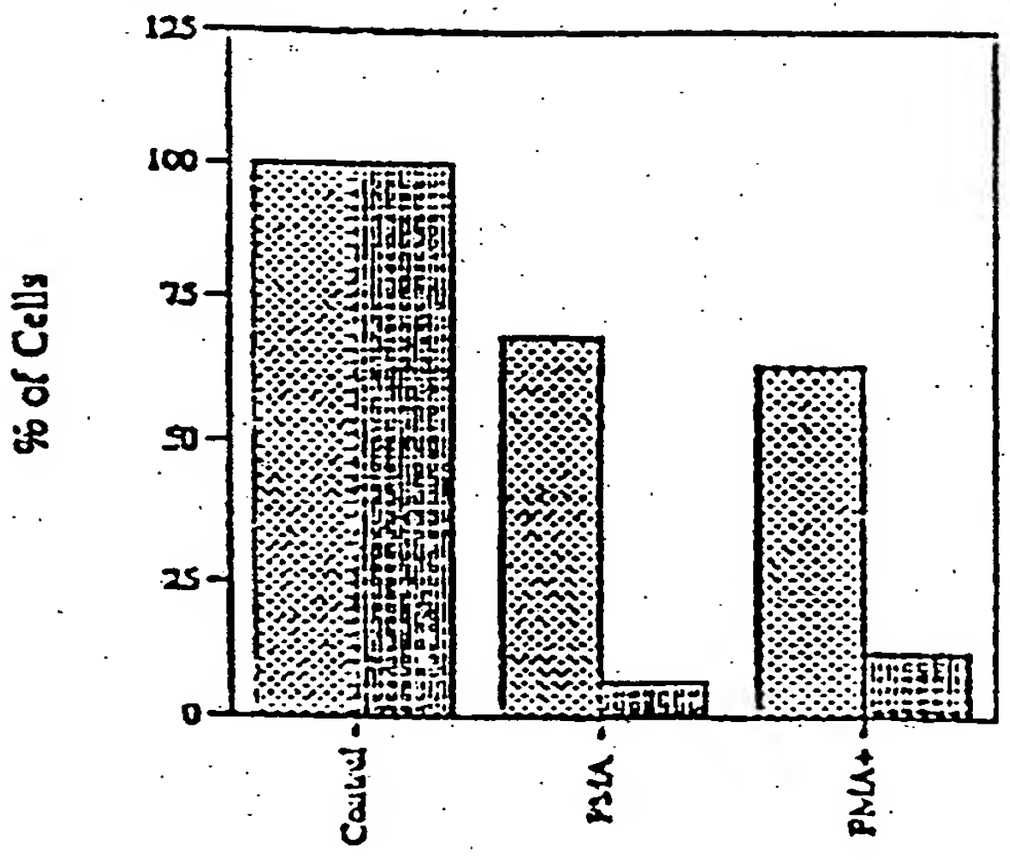
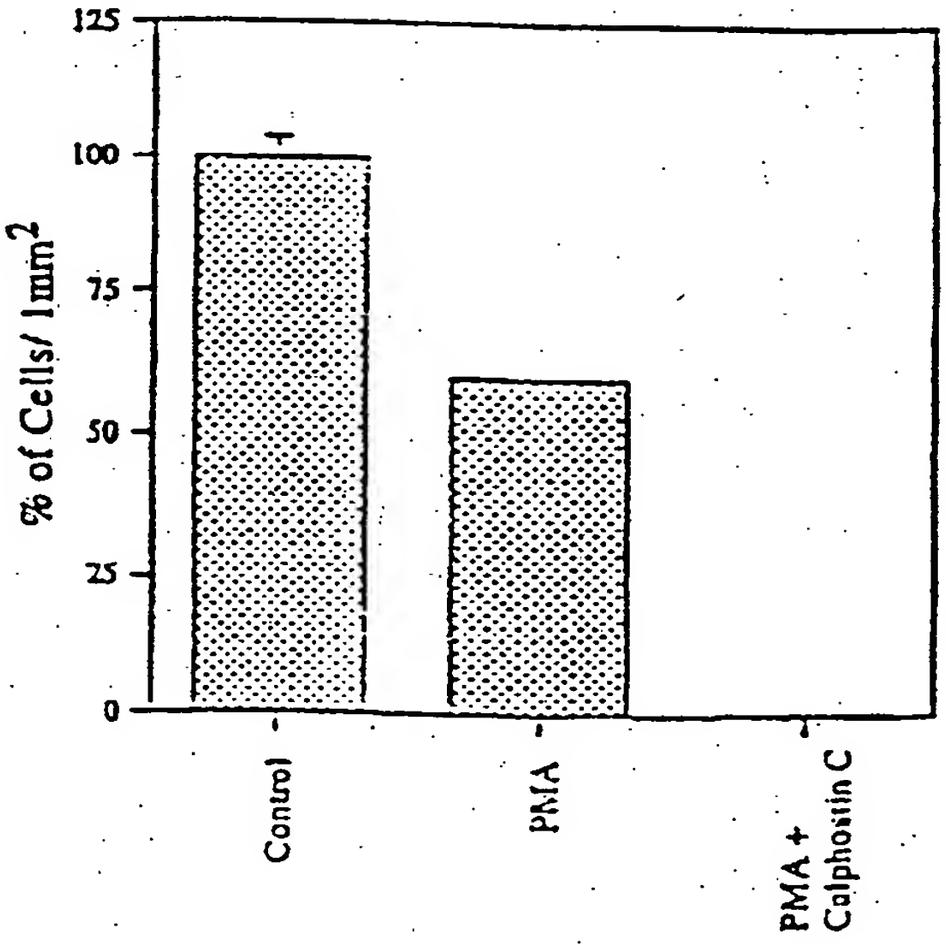


FIG. 9C



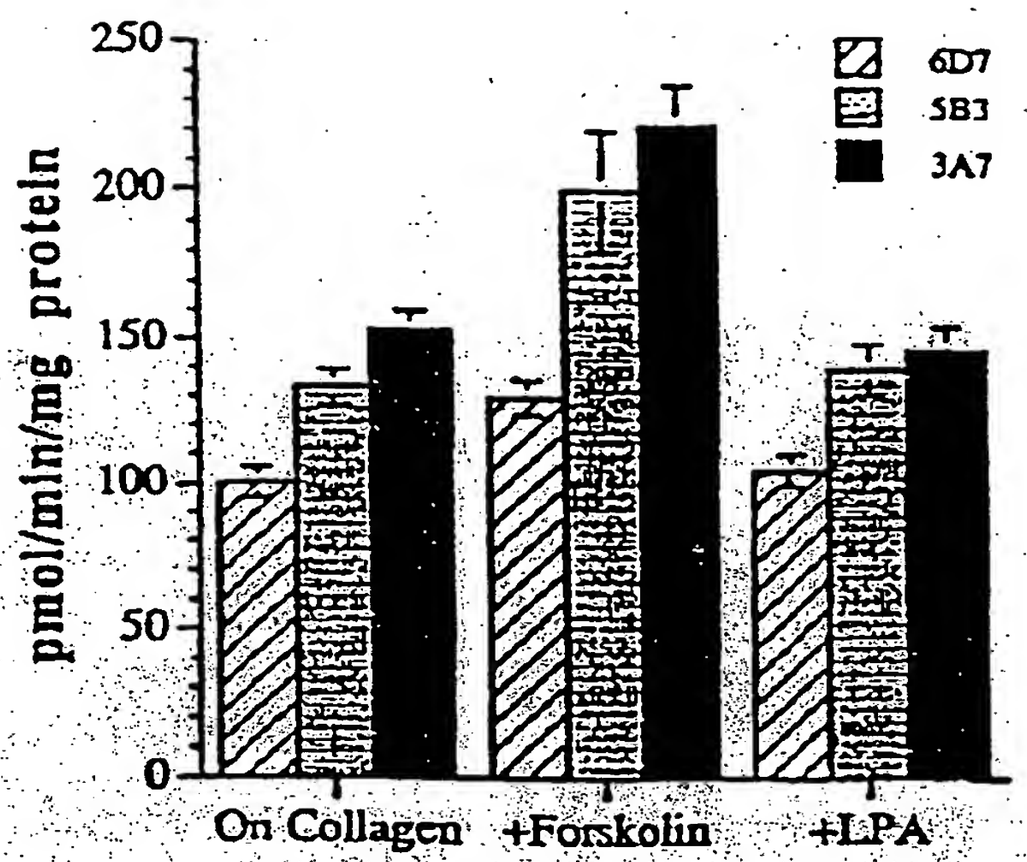


FIG. 10

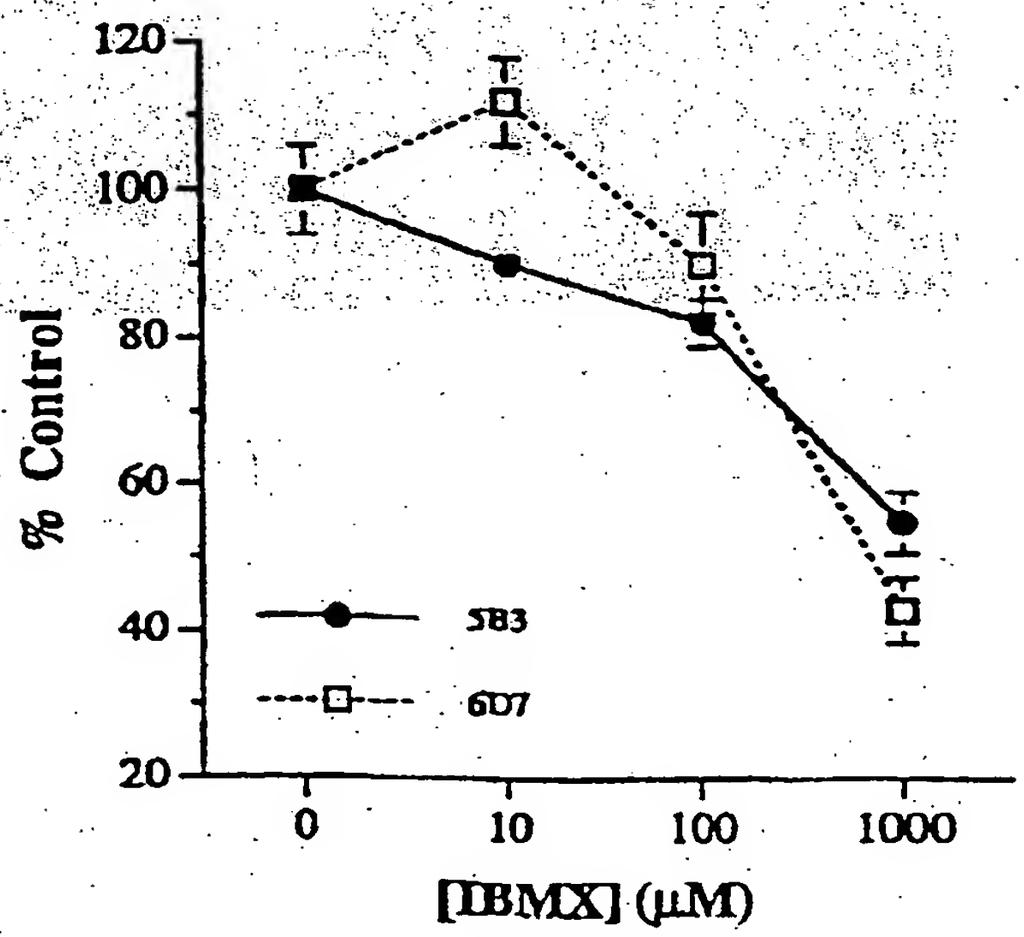


FIG. 11

BNSDOCID: <WO___9935283A1_I_>

FIG. 12A

FIG. 12B

FIG. 12C

FIG. 12D

FIG. 12E

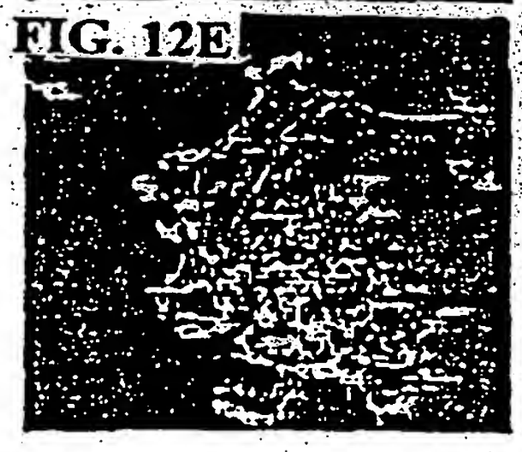


FIG. 12F

BNSDOCID: <WO__9935283A1_I_>

FIG. 13A

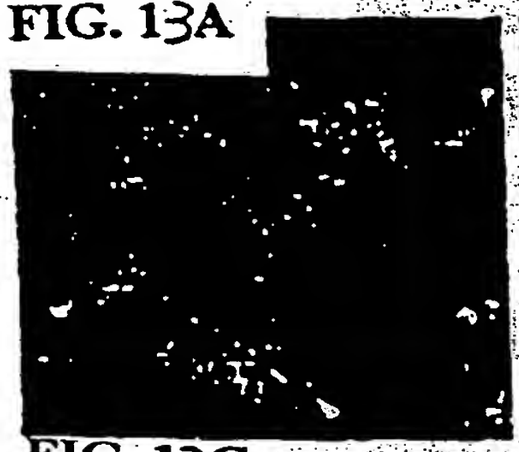


FIG. 13B

FIG. 13C

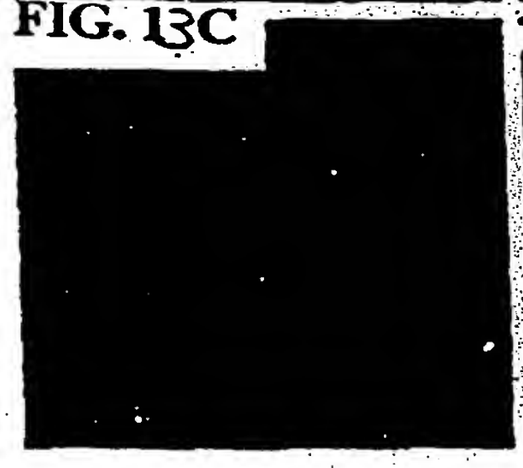


FIG. 13D

BNSDOCID: <WO___9935283A1_I_>

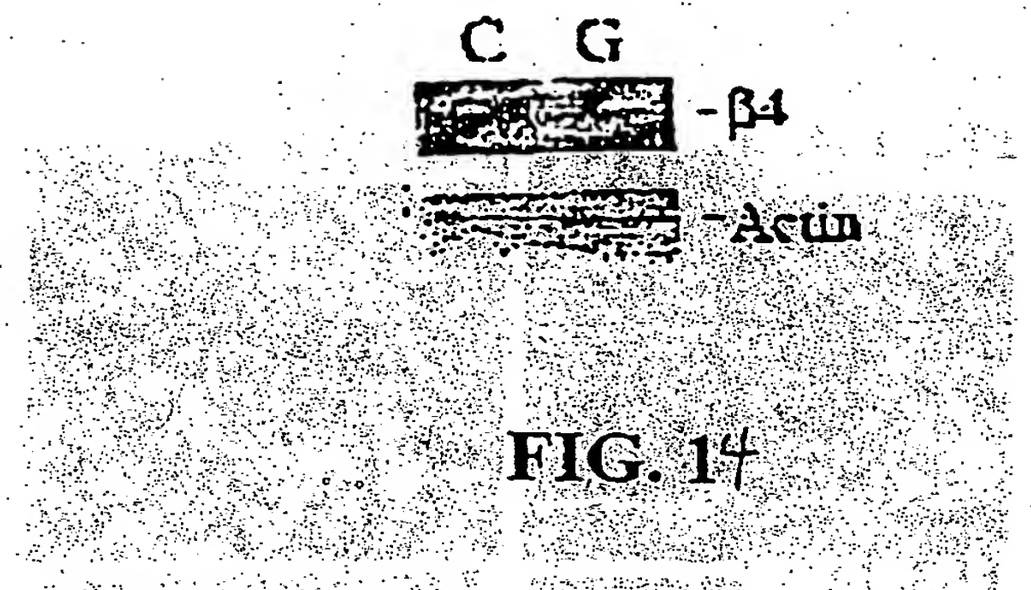


FIG. 14

BNSDOCID: <WO_ _9935283A1_I_>

FIG. 15A

FIG. 15B

FIG. 15C

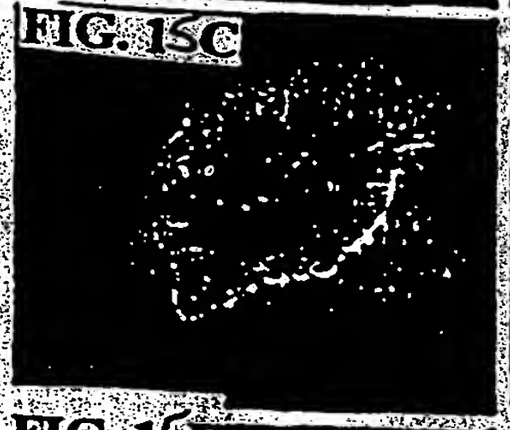


FIG. 15D

FIG. 15E

FIG. 15F

FIG. 16A

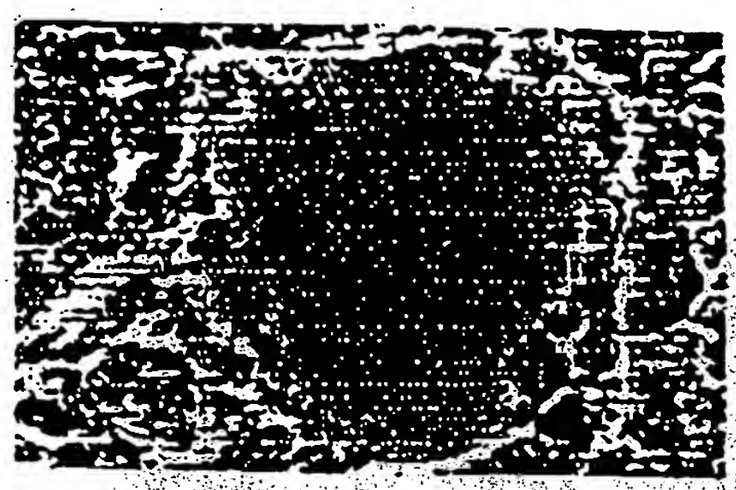


FIG. 16D

FIG. 16B

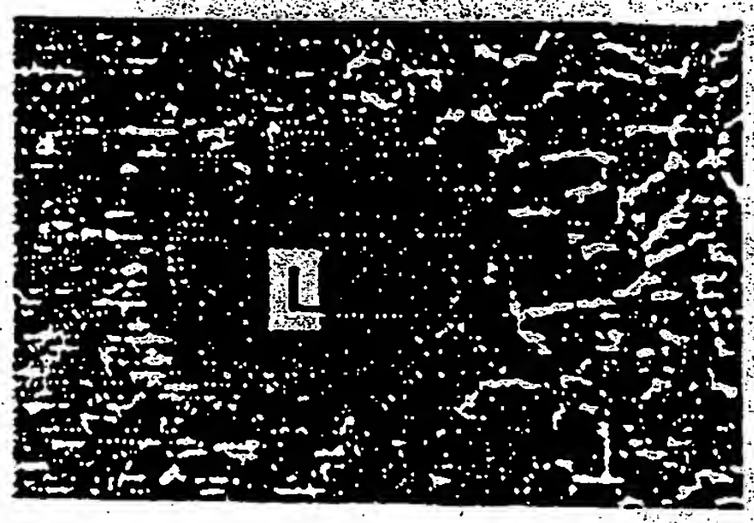


FIG. 16E

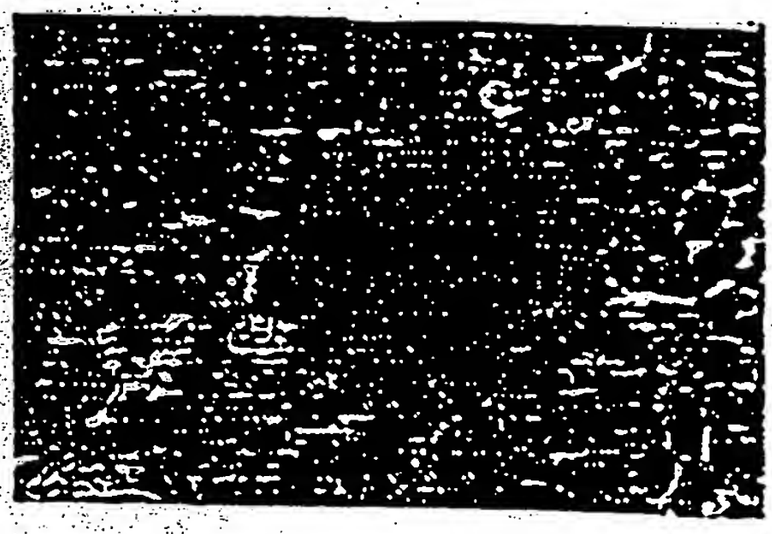


FIG. 16C

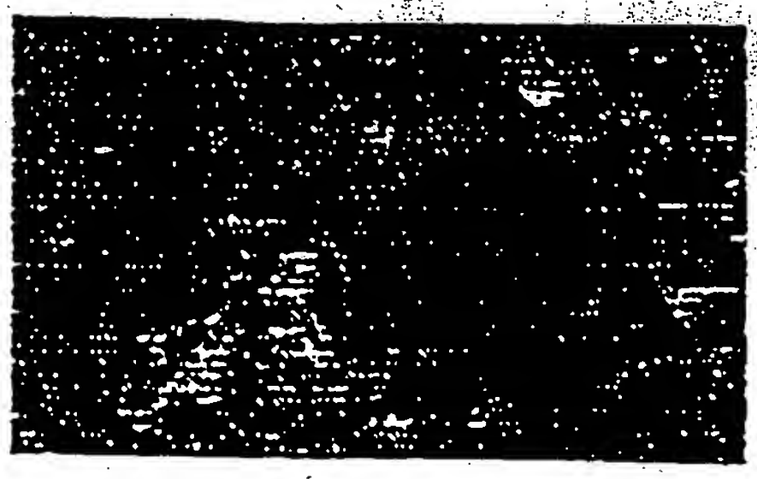
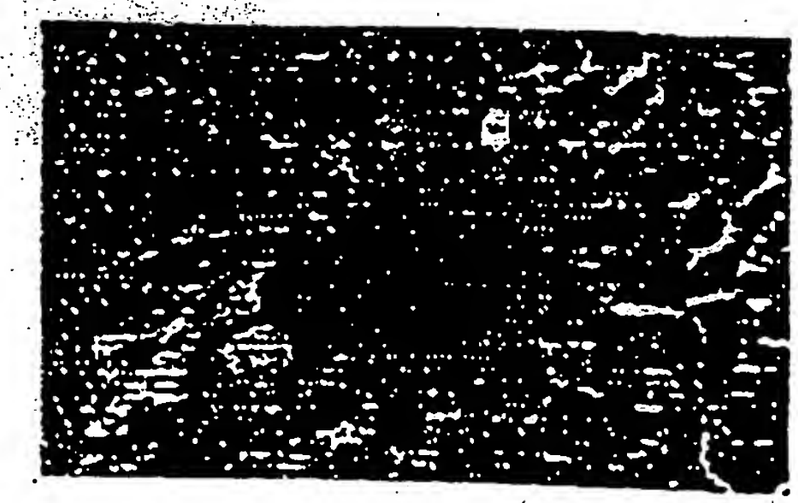


FIG. 16F



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26720

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/25; G01N 33/53
US.CL :435/4, 7.1, 40.5, 69.1; 436/63, 64, 813

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 7.1, 40.5, 69.1; 436/63, 64, 813

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS
search terms: PI3K, phosphoinositide 3 kinase, protein kinase C, integrin, metastasis, motility, cAMP, cyclic AMP

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P -- Y,P	SHAW et al. Activation of phophoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. Cell. 26 December 1997, Vol. 91, No. 7, pages 949-960, see entire document.	1 - 4 , 6 - 9 , 11,13,15,16,18-20, 23 --- 5, 10, 12, 21, 22, 24, 25
X,P --- Y,P	O'CONNOR et al. Release of cAMP gating by the alpha6beta4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. J. of Cell Biology. 14 December 1998, Vol. 143, No. 6, pages 1749-1760, see entire document.	1 - 4 , 6 - 9 , 11,13,15-20, 23 --- 5, 10, 12, 21,22, 24, 25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search
27 MAY 1999

Date of mailing of the international search report
11 JUN 1999

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Form PCT/ISA/210 (second sheet)(July 1992)*

ISDOCID: <WO__9935283A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26720

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database BIOSIS on STN, No. 1998:20487, SHAW et al. The alpha-6 beta-4 integrin promotes carcinoma invasion by activating phosphoinositide 3-kinase. Molecular Biology of the Cell. November 1997, Vol. 8(Suppl), page 183A, see entire document.	1-3, 6-8, 11, 16, 18, 20, 23
X,P	RIGOT et al. Integrin ligation and PKC activation are required for migration of colon carcinoma cells. J. Cell. Science. 1998, Vol. 111(20), pages 3119-3127, especially pages 3119-3121.	7,20, 22, 24
---		---
Y,P		8,9,21,25

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*